ANNE K. HYVÄRINEN

Functional Analysis of the MTERF Protein Family in Cultured Human Cells

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ACADEMIC DISSERTATION
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the Faculty of Medicine of the University of Tampere,
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Palapeli ratkaistaan yksinkertaisesti käymällä
kaikki mahdolliset yhdistelmät läpi yksi kerrallaan.
- Georges Perec: Elämä Käyttöohje

Äidilleni ja Isäni muistolle

Janille ja ihanalle tyttärellemme Ellalle

A jigsaw puzzle is solved simply by going
through all the possible combinations one by one.
- Georges Perec: Life: A User's manual

To my Mother and to the memory of my Father

To Jani and to our wonderful daughter Ella

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This thesis is based on the following articles:

I. Hyvärinen AK*, Pohjoismäki JL, Reyes A, Wanrooij S, Yasukawa T, Karhunen PJ, Spelbrink JN, Holt IJ and Jacobs HT.

The mitochondrial transcription termination factor MTERF modulates replication pausing in human mitochondrial DNA. Nucleic Acids Res 35 (19): 6458-74, 2007. #

II. Hyvärinen AK, Kumanto MK, Marjavaara SK and Jacobs HT.

Effects on mitochondrial transcription of manipulating mTERF protein levels in cultured human HEK293 cells. BMC Mol Biol 11:72, 2010.

III. Hyvärinen AK, Pohjoismäki JL, Holt IJ and Jacobs HT.

Over-expression of MTERFD1 or MTERFD3 impairs the completion of mitochondrial DNA replication. Mol Biol Rep, in press, e-pub June 25, 2010.

IV. Pohjoismäki JL, Wanrooij S, Hyvärinen AK, Goffart S, Holt IJ, Spelbrink JN and Jacobs HT.

Alterations to the expression level of mitochondrial transcription factor A, TFAM, modify the mode of mitochondrial DNA replication in cultured human cells. Nucleic Acids Res 34 (20): 5815-28, 2006. #

This article has been also used in the PhD thesis of Jaakko Pohjoismäki

* Joint first authorship

Abstract

Human mitochondrial DNA (mtDNA) is a double-stranded circular molecule of ~16 kb. In the major coding strand of human mtDNA there are two transcription units, one of which is dedicated to the synthesis of ribosomal RNAs and two transfer RNAs (the 'rRNA transcription unit') and the other one to the synthesis of all messenger RNAs and the remaining transfer RNAs (the 'mRNA transcription unit'). The initiation sites for these two transcription units are located near each other and the transcription units partially overlap. They are independently controlled and differentially expressed. The central aim of the present project was to study the functional roles of human mitochondrial transcription termination factor (MTERF), the protein that is believed to control the relative activities of the two transcription units in the major coding strand of mtDNA.

MTERF is a DNA-binding protein that interacts with mtDNA as a monomer. It binds to a 28 bp region within the leucine (UUR) transfer RNA ($tRNA^{Leu(UUR)}$) gene at the position immediately adjacent and downstream of the 16S ribosomal gene. *In vitro* MTERF has been shown to promote transcription termination but so far no evidence has been reported supporting the idea that it performs such a role *in vivo*. The A3243G MELAS (mitochondrial encephalomyopathy with lactic acidosis and strokelike episodes) mutation is located within the MTERF binding sequence in mtDNA. It has been suggested that elucidating the physiological function(s) of MTERF could help to understand the pathogenesis of MELAS syndrome. It has been shown that the A3243G mutation reduces the binding affinity of MTERF to its target sequence, which should mean that the efficiency of rRNA transcription termination decreases.

MTERF belongs to a family of related proteins whose physiological functions are unclear. This study addressed the issue of the functional role of MTERF and that of two novel MTERF protein family members MTERFD1 and MTERFD3 *in vivo* at the cellular level. The effect of MTERF over-expression and knock down in HEK293T-derived cells was studied on steady-state mitochondrial transcript levels and after mtDNA and RNA depletion with EtBr. Modulating MTERF levels *in vivo* had a modest effect on mitochondrial transcription. It may be inferred that MTERF levels do not determine the relative levels of transcripts representing the two different transcription units of the heavy strand in a simple manner but that compensatory mechanisms are involved. Whereas altering MTERF levels had only minor effects on mitochondrial transcript levels, over-expression of TFAM had a clear effect by slowing down the recovery of the tRNA levels after EtBr-induced depletion of mitochondrial DNA and RNA.

Using two-dimensional neutral agarose gel electrophoresis (2DNAGE), MTERF over-expression or knockdown was found to affect mtDNA replication pausing, although no effect on mtDNA copynumber was detected. MTERF was inferred to promote pausing both at the canonical MTERF-binding site as well as at novel, weaker binding sites identified by electrophoretic mobility shift assay (EMSA) and by using systematic evolution of ligands by exponential enrichment (SELEX). In contrast to MTERF over-expression enhanced replication pause sites, the pause sites enhanced by TFAM over-expression were found comparatively diffuse.

Immunocytochemistry showed that epitope-tagged MTERFD1 and MTERFD3 are mitochondrially targeted, but EMSA and SELEX did not identify plausible sites of sequence-specific DNA binding for either of these proteins. Over-expression of epitope-tagged MTERFD3 or, to a lesser extent, MTERFD1 in HEK293T-derived cells was found to decrease mtDNA copynumber and to impair the completion of mtDNA replication, based on the accumulation of specific classes of replication intermediates, as revealed by 2DNAGE.

In conclusion, the results presented in this thesis further elucidate the role of MTERF in mitochondrial transcription and moreover establish that MTERF has a role also in mtDNA replication. These findings are further analyzed in light of TFAM results. A solid ground for further studies on MTERFD1 and MTERFD3 is laid here as results reported in this thesis indicate that MTERFD1 and MTERFD3 have a role in mtDNA replication too.

Lyhennelmä

Ihmisen mitokondrion DNA (mtDNA) on ~16 kb pitkä kaksijuosteinen rengasmainen molekyyli. Ihmisen mtDNA:n raskaassa koodaavassa juosteessa on kaksi transkriptioyksikköä, joista toinen on dedikoitu ribosomaalisten RNA:iden ja kahden siirtäjä-RNA:n synteesiä varten (’rRNA transkriptioyksikkö’) ja toinen kaikkien lähetti-RNA:iden ja loppujen siirtäjä-RNA:iden synteesille (’mRNA transkriptioyksikkö’). Transkriptioyksiköt menevät osittain päälletysten ja niiden aloituskohdat sijaitsevat lähellä toisiaan. Niitä myös säädellään itsenäisesti ja ilmennetään erillisesti. Ihmisen mitokondriaalisen transkription terminaatiofaktorin (MTERF) oletetaan kontrolloivan mtDNA:n raskaan koodaavan juosteen kahden transkriptioyksikön suhteellisia aktiivisuuksia. Tämän projektin keskeinen tavoite oli tutkia MTERF:n tehtäviä mtDNA:n transkriptiossa ja replikaatiossa.

MTERF on DNA:han sitoutuva proteiini, joka sitoutuu mtDNA:han monomeerinä. Se sitoutuu 28 emäsparin mittaiselle alueelle leusiini (UUR) siirtäjä-RNA:ta ($tRNA^{Leu(UUR)}$) koodaavaan geeniin, 16S koodaavan geenin välittömään läheisyyteen siitä alavirtaan. MTERF:n on *in vitro* osoitettu edistävän transkription terminaatiota, mutta toistaiseksi ei ole näytetty, että sillä olisi vastaava rooli *in vivo*. MELAS-syndrooman (mitokondriaalinen enkefalopatia, laktaattiasidoosi ja kohtausmaiset episodit) aiheuttava A3243G-mutaatio sijaitsee MTERF:n kohdesekvenssissä mtDNA:ssa. On esitetty, että MTERF:n fysiologisten tehtävien selvittäminen voisi auttaa ymmärtämään MELAS-syndrooman syntyä. A3243G-mutaation on osoitettu vähentävän MTERF:n sitoutumisaffiniteettia sen kohdesekvenssiin, jonka pitäisi merkitä sitä, että transkription lopetus rRNA-transkriptioyksikön jälkeen vähenee.

MTERF kuuluu proteiiniperheeseen, jonka muiden jäsenten fysiologiset tehtävät ovat toistaiseksi vielä epäselvät. Tässä projektissa tutkittiin MTERF:n ja kahden uuden MTERF-proteiiniperheen jäsenen, MTERFD1:n ja MTERFD3:n, tehtäviä *in vivo* käyttäen viljeltyjä ihmissoluja. MTERF:n ylituotannon ja vaiennuksen vaikutusta mitokondriaalisiin transkriptitasoihin tutkittiin normaalisti kasvavissa HEK293T-soluissa ja lisäksi EtBr-käsittelyllä aiheutetun mtDNA- ja RNA-depleetion jälkeen. MTERF-proteiinitasojen muuntelu *in vivo* vaikutti vain lievästi mitokondriaaliseen transkriptioon. Tämä implikoi, että MTERF-proteiinin määrä ei määrittele raskaan juosteen eri transkriptioyksiköitä edustavien transkriptien suhteellisia tasoja millään yksinkertaisella tavalla vaan että siihen liittyy kompensaatiomekanismeja. Siinä missä MTERF-proteiinin määrän muutoksella oli vain vähäinen vaikutus mitokondrion transkriptitasoihin, TFAM:n ylituotannolla oli selkeä vaikutus, sillä se hidasti tRNA-tasojen palautumista normaalille tasolle EtBr-käsittelyllä aiheutetun mitokondriaalisen DNA- ja RNA-depleetion jälkeen.

2DNAGE:n (kaksiulotteinen neutraali agaroosigeelielektroforeesi) perusteella MTERF:n ylituotannon sekä vaiennuksen havaittiin vaikuttavan mtDNA:n replikaation taukoamiseen, vaikkei vaikutusta mtDNA:n kopiolukumäärään havaittu. Siksi katsottiin, että MTERF edistää replikaation taukoamista kanoonisessa sitoutumiskohdassaan ja myös uusissa, heikommissa sitoutumiskohdissaan, jotka löydettiin käyttäen EMSA:a (elektroforeettinen liikkuvuudenmuutoskoe) ja SELEX:ä (DNA-ligandien systemaattinen evoluutio eksponentiaalisella rikastuksella). TFAM:n voimistamat replikaation pysäytyskohdat olivat varsin diffuuseja toisin kuin MTERF:n vastaavat.

Immunosytokemia osoitti, että epitooppi-merkityt MTERFD1 ja MTERFD3 ovat mitokondriaalisesti kohdennettuja proteiineja, mutta EMSA:n ja SELEX:n keinoin ei löydetty sekvenssispesifejä sitoutumiskohtia kummallekaan näistä proteiineista. Epitooppi-merkityn MTERFD3:n ja hieman vähemmissä määrin MTERFD1:n ylituotannon havaittiin vähentävän mtDNA:n kopiolukumäärää ja estävän mtDNA:n replikaation loppuunsaattamista HEK293T-johdetuissa soluissa perustuen 2DNAGE:lla havaittuun tiettyjen replikaatiovälituotteiden lisääntyneeseen määrään.

Yhteenvetona tässä väitöskirjassa esitetyt tulokset valottavat MTERF:n roolia mitokondriaalisessa transkriptiossa ja osoittavat, että MTERF vaikuttaa myös mtDNA:n replikaatiossa. Näitä löydöksiä analysoidaan myös TFAM-tutkimuksen tulosten valossa. Tämä tutkimus luo hyvän pohjan MTERFD1:n ja MTERFD3:n jatkotutkimukselle, sillä nyt raportoidut tulokset osoittavat, että niillä mahdollisesti on rooli mtDNA:n replikaatiossa.

List of abbreviations

2DNAGE	two-dimensional neutral agarose gel electrophoresis
aa	amino acid
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BSA	bovine serum albumin
bp	basepair
cDNA	complementary DNA
CPEO	chronic progressive external ophtalmoplegia
DHU	dihydrouridine
D-loop	displacement loop
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
DTT	dithiotreithol
EMSA	electrophoretic mobility shift assay
EtBr	ethidium bromide
FCS	fetal calf serum
HMG	high mobility group
hsDNA	herring sperm DNA
HSP	heavy strand promoter
H-strand	heavy strand
HUGO	human genome organization
IM	inner membrane
IMS	inter-membrane space
KSS	Kearns-Sayre syndrome

LB-medium	Luria Bertoli medium
LHON	Leber's hereditary optic neuroretinopathy
LM	n-dodecyl β-D-maltoside
LSP	light strand promoter
L-strand	light strand
MELAS	mitochondrial myopathy, encephalopathy, lactic acidosis and stroke like episodes
MERRF	myoclonic epilepsy and ragged-red fibres
mRNA	messenger RNA
mt	mitochondrial
mtDNA	mitochondrial DNA
MTERF	mitochondrial transcription termination factor
MTERFD1	mitochondrial transcription termination factor D1
MTERFD3	mitochondrial transcription termination factor D3
mtRI	mitochondrial replication intermediate
mtRNA	mitochondrial RNA
mtRPOL	mitochondrial RNA polymerase
mtTFA	mitochondrial transcription factor A
mtTFB	mitochondrial transcription factor B
NARP	Neuropathy, ataxia and retinis pigmentosa
NCR	non-coding region
ND	NADH dehydrogenase
nt	nucleotide
np	nucleotide position
OE	over-expressor
O_H	heavy strand origin
O_L	light strand origin
OM	outer membrane
OXPHOS	oxidative phosphorylation
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction

PBS	phosphate buffered saline
PE	progressive encephalopathy
PEO	progressive external ophthalmoplegia
PH1	heavy strand transcription initiation site 1
PH2	heavy strand transcription initiation site 2
PL	light strand transcription initiation site
PMSF	phenyl-methyl-sulfonyl-fluoride
POLG	DNA polymerase γ
POLRMT	mitochondrial RNA polymerase
Q-RT-PCR	quantitative RT-PCR
RI	replication intermediate
RITOLS	RNA incorporation throughout the lagging strand
RNA	ribonucleic acid
RNAi	RNA interference
RNase MRP	mtRNA-processing endoribonuclease
rRNA	ribosomal RNA
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecylsulphate
SELEX	systematic evolution of ligands by exponential enrichment
siRNA	short interfering RNA
SSB	single strand binding
SSC	sodium-chloride sodium citrate buffer
ssDNA	single strand DNA
TAP	thiamphenicol
TBE	tris-borate-EDTA
TERM	transcription termination sequence
TFAM	mitochondrial transcription factor A = mtTFA
TFB1M	mitochondrial transcription factor B1
TFB2M	mitochondrial transcription factor B2
Top1mt	mitochondrial topoisomerase

tRNA	transfer RNA
u	unit
u.v.-light	ultra violet light
vol	volume
w/v	weight/volume

1. Introduction

Mitochondria have evolved from a symbiotic relationship between an ancestral eukaryote cell lacking mitochondria and an aerobic eubacterium (proteobacterium) capable of oxidative phosphorylation, as described by Margulis (1981). Nowadays this commonly accepted endosymbiosis theory considers that the protoeukaryote has internalized the simpler proteobacterium, which then evolved into mitochondria (Gray *et al.* 1999, Andersson *et al.* 2002). From those early days mitochondria have evolved to be membrane-bound cell organelles with a genome and genetic code of their own.

The most important function of mitochondria is to release energy from carbohydrates, fatty acids and amino acids to be used by the cells, and thus mitochondria are sometimes called the power plants of cells. The respiratory chain is located in the inner mitochondrial membrane where the final steps of energy conservation take place (Scheffler 1999). It has been established that mitochondria have other functions, in addition to energy production. Mitochondria are generally needed for proper cell function since they have various tasks in building, breaking down and recycling molecules within the cell. Mitochondria function in heat production as reviewed by Watanabe *et al*. (2008), they serve as a storage for calcium and play a role in calcium signalling as well as have a role in regulating membrane potential (Graier *et al*. 2007). Mitochondria have a role in cell metabolism and are required for biosynthesis of heme (Schultz *et al*. 2010) and steroids (Sewer and Li 2008) and then again in liver in metabolic detoxification of ammonia in urea cycle (Campbell 1997). Mitochondria also have a role in cell proliferation (Merkwirth and Langer 2009). They have been reported to be involved in apoptosis (Desagher and Martinou 2000, Sastre *et al.* 2000, Kar *et al*. 2010) and mutations occurring in the mitochondrial genome have been suggested to have a role in the pathogenesis of many diseases (Jacobs 1997, Taylor and Turnbull 2005, Copeland

2010). Mitochondrial diseases are a broad range of diseases that are due to various point mutations and genome rearrangements occurring in the mitochondrial genome (Suomalainen 1997, Zeviani 2004) or in nuclear genes coding for mitochondrial proteins. Mitochondrial dysfunction is suggested to have a role in type 2 diabetes (Wang *et al.* 2010) and in many neurogenerative diseases as well as in cancer (de Moura *et al*. 2010). Recently mitochondria were established to have a role in metastasis, as ROS scavengers were found to be therapeutically effective in suppressing metastasis (Ishikawa and Hayashi 2010). Also more and more data is published supporting mitochondria having a role in aging (Jacobs 2003, Trifunovic *et al.* 2004, Sanz *et al*. 2010b). However, such a role is not necessarily a direct one, as in *Drosophila* mitochondrial ROS production was found to correlate with lifespan but not to regulate it (Sanz *et al*. 2010a).

Mitochondria have their own genome and even use a variant of the genetic code, making them different from other mammalian cell organelles (Barrell *et al.* 1979, Anderson *et al.* 1981). This is due to the evolution of the mitochondria following the symbiosis of the protoeukaryote and the proteobacterium. This evolutionary background makes mitochondrial transcription and replication processes interesting fields of research. Different models have been presented to describe mitochondrial DNA replication, discussing whether it resembles more its nuclear or bacterial equivalent.

In the following literature review the mechanisms of mitochondrial DNA replication and transcription, the proteins of the mitochondrial transcription termination factor (MTERF) family and their possible roles in human mtDNA maintenance are discussed.

2. Literature review

2.1 Mitochondria

2.1.1 Structure of mitochondria

Mitochondria are membrane bound cell organelles dedicated to energy production. Historically from the 1950s up until the 1990s it was considered that mitochondria are formed from two highly specialized membranes, the inner (IM) and outer membrane (OM) and that the inner membrane forms cristae by infolding. This is also referred to as the ‘baffle model’ of mitochondrial structure (Palade 1952).

Nowadays, the prevailing opinion is that mitochondria consist of at least 6 compartments, namely the outer membrane, the inner boundary membrane, the space between the two membranes called the intermembrane space, the cristal membranes, the intracristal space and the space inside the inner membrane which is called the mitochondrial matrix (Perkins *et al.* 1997, Frey and Mannella 2000, Logan 2006). The cristal membranes form lamellar structures. These are connected to the inner boundary membrane by small tubular structures called crista junctions (Perkins *et al.* 1997). Sites, where the inner boundary membrane comes into close contact with the outer membrane, are known as contact sites. At these numerous contact sites there are protein translocation pores through which the transportation of proteins from the cytosol to the matrix is effected (Schatz and Dobberstein 1996).

2.1.2 The mitochondrial network

Mitochondria are present in all human cells with few exceptions (essentially only mature red blood cells). Different cells have a different degree of energy demand and this determines the number and the shape of the mitochondria in each cell type. Mitochondria form a dynamic network inside the cell, which can be considered as a reticulum characterized by constant fusion and fission of the mitochondria (Bereiter-Hahn and Voth 1994, Nunnari *et al.* 1997), affected by many proteins (Thomson 2002). Mitochondria are also attached to the cytoskeleton and it is established that cytoskeleton has an important role in mitochondrial and cell morphology (Anesti and Scorrano 2006). In mammalian cells the precise distribution of the mitochondria appears to be organized by the microtubular network, and is modulated by many connector and motor proteins (for review see Vale 2003, Hollenbeck and Saxton 2005). Intracellular transportation of mitochondria is necessary if more mitochondria are required in certain part of the cell due to increased energy demand or if a mitochondrion is to be degraded as reviewed by Hollenbeck and Saxton (2005). Also, mitochondrial dynamics and organization within a cell is highly cell-type specific, indicating the importance of interactions between mitochondria and other intracellular compartments. Mitochondria are comparatively immobile for example in adult rat cardiomyocytes where they present low amplitude fluctuation or vibration (Beraud *et al*. 2009); conversely they are very mobile inside neurons or pancreatic cells, showing complex dynamics including fission, fusion, oscillating movement and even rapid long-distance migration as reviewed by Boldogh and Pon (2007). It remains to be studied which proteins mediate the attachment between mitochondria and microtubules. Also the role of these proteins, if any, in organizing mtDNA remains to be elucidated.

2.1.3 The human mitochondrial genome

Mitochondrial DNA – or the rho factor as it was then called - was first reported in the 1940's. Nass and Nass (1963) were the first who were able to detect chicken mtDNA using microscopy. In the course of metazoan evolution the mitochondrial genome has

gradually shortened to vary between 15-20 kb. Spacer sequences have almost completely disappeared and most of the 'original' mitochondrial genes have been transferred to the nuclear genome, which may facilitate mtDNA replication and make it less error-prone. Human mtDNA, as illustrated in Figure 2.1, is a 16 568 bp long circular, double-stranded DNA molecule (Anderson *et al.* 1981, Andrews *et al.* 1999).

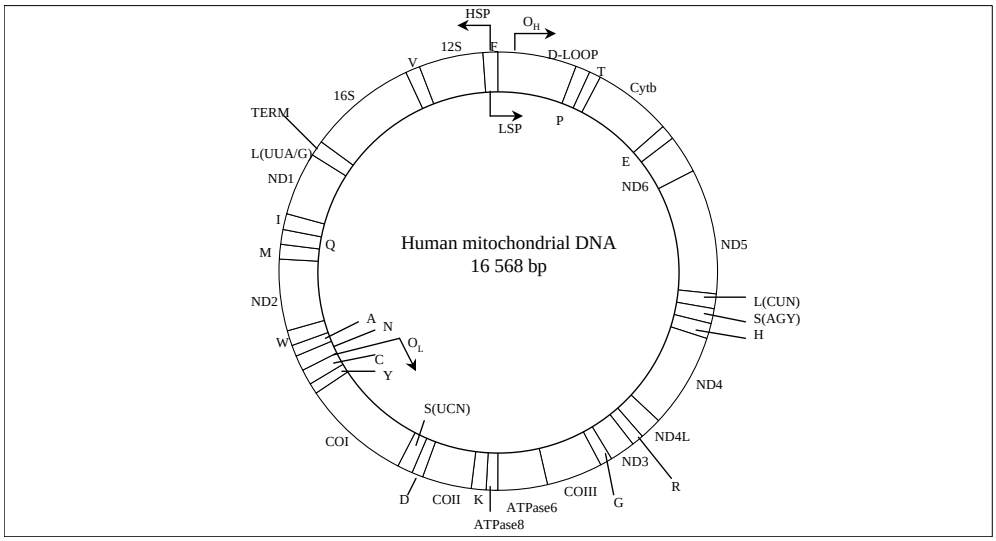

Figure 2.1. Human mitochondrial DNA (Anderson *et al.* 1981). Transfer RNAs are denoted in the one letter amino-acid code and in addition L(UUA/G) = $tRNA^{Leu(UUR)}$, L(CUN) = $tRNA^{Leu(CUN)}$, S(UCN) = $tRNA^{Ser(UCN)}$ and S(AGY) = $tRNA^{Ser(AGY)}$. 12S = 12S ribosomal RNA, 16S = 16S ribosomal RNA, ND1-6 = NADH dehydrogenase 1-6, COXI-III = Cytochrome c oxidase I - III, ATPase 8 = ATP synthase subunit 8, ATPase 6 = ATP synthase subunit 6, Cytb = apocytochrome b.

2.1.4 Organization of the human mitochondrial genome

13 out of the 37 human mitochondrial genes encode protein subunits needed in oxidative phosphorylation. ND1-6 and ND4L encode subunits of NADH dehydrogenase (complex I) of the respiratory chain. Apocytochrome b encodes a protein subunit of the *bc1* complex (complex III), COXI-III encode subunits of the cytochrome c oxidase (complex IV) and ATP synthase subunits 6 and 8 are part of the ATP synthase complex (complex V) (Anderson *et al.* 1981, Chomyn *et al.* 1985). Two genes encode ribosomal RNA

(rRNA) molecules needed for mitochondrial translation. mtDNA also contains the tRNA-encoding genes needed for mitochondrial translation, although nuclearly encoded tRNA has been also shown to be imported to mitochondria under some conditions. Suyama (1967) first reported nucleus-encoded tRNA present in *Tetrahymena pyroformis* mitochondria. According to Tarassov *et al*. (2007) mitochondria import tRNAs to compensate for any lack of mitochondrial tRNAs. For example the marsupial mitochondrial $tRNA^{Lys}$ gene is actually a pseudogene, the functional $tRNA^{Lys}$ being imported from the cytosol (Dörner *et al.* 2001). Cytosolic $tRNA^{Gln(CUG)}$ and $tRNA^{Gln(UUG)}$ are also imported in yeast and human mitochondria (Rinehart *et al*. 2005, Rubio *et al*. 2008) even if the mitochondrial $tRNA^{Gln(UUR)}$ is expressed and able to read the CAA and CAG codons (Maréchal-Drouard *et al*. 1993). This kind of redundancy found in yeast and human mitochondria cannot yet be explained.

2.1.5 Mitochondrial nucleoids and inheritance of mtDNA

According to present knowledge mitochondrial DNA is organized in nucleoids as reviewed by Spelbrink (2010). One nucleoid has been reported to contain typically 2-10 mtDNA molecules (Iborra *et al*. 2004, Legros *et al*. 2004). mtDNA has been shown to be packed with proteins as dynamic nucleoids (Garrido *et al.* 2003, Alam *et al.* 2003) in which it is wrapped with TFAM proteins. Spelbrink *et al*. (2001) reported that the human Twinkle helicase colocalizes with mtDNA and Garrido *et al*. (2003) showed that mtDNA polymerase POLG also copurifies with mtDNA nucleoids. The list of confirmed nucleoid proteins currently includes the human DEAH helicase DHX30 (Wang and Bogenhagen 2006), the protein designated M19 (Sumitami *et al*. 2009), the DNA binding protein ATAD3 (He *et al*. 2007), Dna2 (Duxin *et al*. 2009) and recently MTERFD3 (Pellegrini *et al*. 2009). mtSSB, mitochondrial single stranded DNA binding protein, is required for maintenance of mtDNA but not for mitochondrial nucleoid organization (Ruhanen *et al*. 2010). mtDNA is often erroneously referred to as ‘naked’ which is clearly not the case.

To explain the organization and maintenance of mtDNA in mammalian somatic cells Jacobs *et al.* (2000) proposed that a group of mtDNA molecules known as a mitochondrial nucleoid form the unit of genetic function. The mtDNA molecules comprising one nucleoid can also be diverse genetically. Jacobs *et al.* (2000) suggested that a nucleoid replicates as a unit and that the genetically identical daughter nucleoids segregate in a manner resembling mitosis when the nucleoid finally divides. The slow rate of mitotic segregation in cultured heteroplasmic cell-lines could be explained by this model, although there is no direct experimental evidence to support it.

Nuclear genes are inherited in Mendelian fashion, one allele from each parent. The mitochondrial genome on the other hand is inherited only from the mother (maternal inheritance). Shitara *et al*. (2000) reported that mitochondria of spermatozoa enter the egg but are soon after destroyed or inactivated during embryonic development (i.e. they do not affect the zygote genetically). Schwartz and Vissing (2002) reported the first observed case of paternal inheritance (i.e. paternal leakage of mtDNA). The case reported was a patient suffering from a mitochondrial myopathy resulting from a 2 bp deletion in the ND2 gene of mtDNA. The authors found out that this mutation was of paternal origin and was present in 90% of the patient's muscle mtDNA (Schwartz and Vissing 2002). Later on Kraytsberg *et al*. (2004) reported recombination of maternal and paternal human mitochondrial DNA in this same patient. However, to date this remains the sole such case.

When all mtDNA molecules in a mitochondrion or in a cell are identical, the situation is referred to as homoplasmy, whereas the opposite situation is referred to as heteroplasmy. When mtDNA is heteroplasmic random mitotic segregation has been proposed to cause variation in mitochondrial genotype between tissues (Macmillan *et al.* 1993, Shoubridge 2000). It was long ago suggested that during oogenesis and early embryogenesis there is little or no selection against pathogenic mtDNA mutations, and a genetic bottleneck was proposed to explain the rapid selection of mtDNA genotype. Jenuth *et al*. (1996) showed that, in murine cells, mtDNA genotype varied less in primordial germ cells than in primary or mature oocytes and therefore the genetic

bottleneck was suggested to cause selection early in the developing oocyte. Individuals could therefore finally carry different amounts of mutant and normal mtDNAs in different tissues and family members suffering from the same mitochondrial disease may thus present very different phenotypes (mitochondrial diseases are discussed in more detail in section 2.1.6 below). Wai *et al*. (2008) finally showed that, during folliculogenesis, some nucleoids are actively replicated whereas some are not and this correlates with an increase in the mtDNA genotype variance in the primary or mature oocytes.

2.1.6 Diseases caused by mutations in mitochondrial DNA

Human disorders rising from mutations in the mitochondrial genome were first reported in the late 1980s (Holt *et al*. 1988, Wallace *et al*. 1988a, Wallace *et al*. 1988b). They may be grouped by their target gene or the type of the mutation.

2.1.6.1 Point mutations in mitochondrial protein coding genes

The first group consists of inherited and often heteroplasmic point mutations in mitochondrial protein coding genes. Leber's hereditary optic neuropathy (LHON) is caused by mutations in genes encoding complexes I or III (Wallace *et al.* 1988a, Brown *et al.* 1992). The disease is characterized by the destruction of the optic nerve, leading to rapid bilateral loss of central vision during adolescence.

2.1.6.2 mtDNA rearrangements

Partial mtDNA deletions or duplications (mtDNA rearrangements) cause changes in the relative content of mitochondrial genes and create abnormal gene junctions. Such mutations are sporadic i.e. they occur with no inheritance. Holt *et al*. (1988) found deletions in the muscle cell mtDNA of patients suffering from different types of mitochondrial myopathy. mtDNA depletion is characterized by a reduced amount of mtDNA molecules, as reviewed by Suomalainen and Isohanni (2010). The associated

disease mechanism is greatly affected by mtDNA replication and nucleotide pool regulation (Suomalainen and Isohanni 2010). Depleted mtDNA and multiple mtDNA deletions have been found to be autosomally inherited but they occur also as sporadic cases. The major phenotype, PEO (progressive external ophthalmoplegia) manifests as ragged-red fibers in the muscle together with ptosis and external ophtalmoplegia (Laforet *et al.* 1995). PEO is often caused by multiple deletions of mtDNA (Zeviani *et al.* 1989).

Some patients carrying mtDNA deletions show only progressive external ophthalmoplegia (PEO) (Schon *et al.* 1997) whereas others may have Kearns-Sayre syndrome (KSS). KSS is a severe disease affecting many tissues and organs, the features often including PEO, ragged-red fibres, ataxia, heart symptoms, mental retardation and dwarfism. The patient is often affected before the age of 20 years (Schon *et al.* 1997), and clinical features differ greatly between patients, which makes the diagnosis of such diseases rather demanding for a physician.

2.1.6.3 Point mutations in mitochondrial tRNA and rRNA genes

Third group of mitochondrial mutations are point mutations in tRNA and rRNA coding genes. More than 70 mutations have been found in tRNA genes (Brandon *et al.* 2005). In addition, a well documented mutation in an rRNA coding gene has been reported, namely the A1555G mutation in 12S rRNA, which is associated with maternally inherited non-syndromic deafness and aminoglycoside-induced deafness (Prezant *et al.* 1993). Regarding non-syndromic deafness, the nuclear genetic background affects the phenotypic expression of the A1555G mutation (Guan *et al.* 2001).

Different mutations in the same tRNA gene and also mutations in different tRNA genes may cause different diseases. In addition, individuals within one family carrying the same mutation may present phenotypes differing dramatically from each other. Mutations in mitochondrial tRNA coding genes cause a wide range of diseases. Goto *et al.* (1990), Kobayashi *et al.* (1990) and Goto *et al.* (1991) first reported that certain mutations in the $tRNA^{Leu(UUR)}$ encoding gene cause MELAS syndrome (mitochondrial

encephalomyopathy with lactic acidosis and strokelike episodes). The same mutation also causes maternally inherited diabetes mellitus +/- deafness, mitochondrial myopathy, CPEO (chronic progressive external ophtalmoplegia) and PE (progressive encephalopathy) in different patients (Brandon *et al.* 2005). $tRNA^{Lys}$ mutations cause MERRF syndrome (myoclonus epilepsy and ragged red fibers) (Wallace *et al.* 1988b, Shoffner *et al.* 1990) whereas $tRNA^{Ser}$ mutations cause maternally inherited sensorineural deafness and ataxia (Tiranti *et al.* 1995). In addition, many other point mutations found in tRNA coding genes are pathogenic with a variety of clinical phenotypes.

2.1.6.4 MELAS mutations

The A>G transition at np 3243 within the $tRNA^{Leu}$ gene responsible for decoding UUR (R = A or G) leucine codons ($tRNA^{Leu(UUR)}$) is the most common mutation causing the MELAS syndrome. Up to 80% of MELAS patients carry the A3243G mutation (Goto *et al.* 1990, Kobayashi *et al.* 1990). Approximately 10% of MELAS patients have a T>C transition mutation at np 3271 (Goto *et al.* 1991). The remaining 10% of MELAS patients carry other mutations in the $tRNA^{Leu(UUR)}$ gene or in some other gene. In the $tRNA^{Leu(UUR)}$ coding gene the A3243G mutation site is located in the dihydrouridine (DHU) loop and the T3271C mutation in the anticodon stem. Interestingly, the clinical phenotypes are the same even if the two mutations are located in different areas of the tRNA.

Pavlakis *et al.* (1984) first described the MELAS syndrome. If mutated mtDNA is present at a relatively low percentage, the patient manifests only type II diabetes, which in some cases can occur with deafness (van den Ouweland *et al.* 1992). However, some patients can carry high levels of mutant mtDNA yet only show mild or tissue-restricted phenotypes, such as diabetes/deafness or PEO. This variability remains unexplained. It is generally considered that the different patient phenotypes are affected also by nuclear genes, in addition to the heteroplasmy level and the distribution of the mutant mtDNA in different tissues.

One mutation can cause clinical phenotypes that differ greatly from each other in different families, indicating that the relative amount of mutant mtDNA does not fully explain all of the variation (van den Ouweland *et al.* 1992, Lightowlers *et al.* 1997). When studying the distribution of the A3243G mutation Chinnery *et al.* (1999) tested whether the mutation percentage of different tissues arises from a totally random process. They found out instead that it segregates in a non-random fashion. This finding suggests a nuclear impact on mtDNA segregation. Battersby *et al.* (2003) reported evidence for the nuclear control of mtDNA segregation in the mouse.

2.2 mtDNA transcription

The two strands of the mitochondrial DNA are named as heavy (H-strand) and light (L-strand) strands as shown below in Figure 2.2. The nomenclature is due to the strands showing different buoyant densities under cesium chloride density gradient centrifugation, since the heavy strand is purine rich and the light strand purine poor. Each strand has one promoter, designated thus as the H-strand promoter (HSP) and the L-strand promoter (LSP). The light and heavy strands are transcribed in opposite directions. Most of the genes are transcribed from the heavy strand. 10 mRNAs, 2 rRNAs and 14 tRNAs of the heavy strand are part of two polycistronic transcripts and only 1 mRNA and 8 tRNAs are encoded by the light strand, as illustrated in Figure 2.1. Genes encoding ATPase 8 + ATPase 6 and ND4L + ND4 are translated from bicistronic mRNAs. Only part of the light strand is coding, but nevertheless it is transcribed almost completely. Chang and Clayton (1984) first identified the independent promoter sequences for both heavy and light strand transcription using *in vitro* assays.

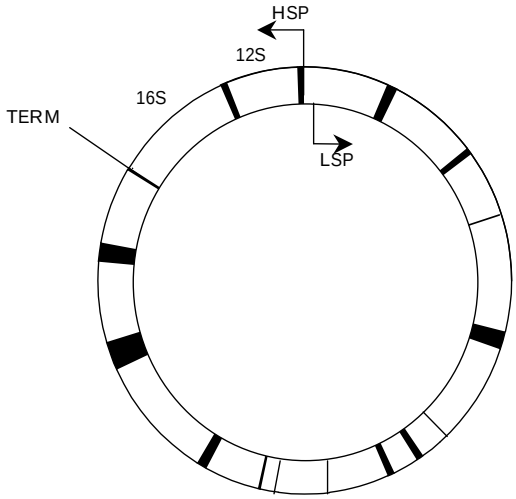

Figure 2.2. The two strands of the mitochondrial genome are called the heavy and light strand. HSP = heavy strand promoter, LSP = light strand promoter, 12S = 12S ribosomal RNA, 16S = 16S ribosomal RNA, TERM = transcription termination sequence. See Figure 2.1. for more detailed description of the human mtDNA functional loci.

Human mitochondrial DNA is packed very tightly and economically as there are virtually no non-coding sequences between genes i.e. human mtDNA lacks introns and spacers. Genes are packed so tightly that some of the genes partially overlap. The polycistronic preliminary transcript therefore has to be post-transcriptionally processed to obtain the final transcription products. According to Reichert *et al.* (1998) one tRNA containing the common base is cleaved out first when processing two adjacent tRNAs sharing a common base. Subsequently the remaining tRNA, now lacking one base, is edited to form the final transcript. Quoting Börner *et al*. (1997), tRNA editing is common in many organisms. In addition to the 37 genes in the human mtDNA there is a ~1 kb long non-coding (D-loop) region (D-loop = displacement loop) that is needed for initiation of mtDNA replication and transcription (Anderson *et al.* 1981, Shadel and Clayton 1997).

2.2.1 Proteins needed for transcription initiation

The mitochondrial transcription machinery is a rather simple system, consisting of mitochondrial RNA polymerase, the core protein, mitochondrial transcription factor A

(TFAM) which acts as an activator, and TFB1M or TFB2M which are needed for initiation. TFB2M has been shown to be primarily the transcription factor (Cotney *et al.* 2007). In 1985 it was reported that a transcription factor (or factors) is needed for specific initiation of transcription at HSP and LSP (Fisher and Clayton 1985) and the same year Hixson and Clayton (1985) established that specific residues at the transcription initiation sites are needed for transcription initiation from either HSP or LSP. Subsequently Fisher *et al.* (1987) established that binding of a transcription factor to a regulatory element, independent of orientation, is required for successful promoter selection. The relevant factor, human TFAM protein of 24.4 kDa, was finally purified and characterized in 1988 (Fisher and Clayton 1988). Recently, Shutt *et al.* (2010) reported that specific transcription initiation can take place *in vitro* independent of TFAM from both LSP and HSP1.

2.2.1.1 Mitochondrial RNA polymerase

Mitochondrial RNA polymerase activity was first characterized by Shuey and Attardi (1985). Masters *et al.* (1987) first established the homology between the yeast mitochondrial RNA polymerase and those of bacteriophages T3 and T7 whereas no homology was detected between the yeast mitochondrial enzyme and *E. coli* RNA polymerase. Tiranti *et al.* (1997) identified the nuclear gene on chromosome 19p13.3. coding for the human mitochondrial RNA polymerase (h-mtRPOL, here called POLRMT) which is a protein of 1230 amino acids. Prieto-Martin *et al.* (2001) suggested that additional factors are needed for transcription initiation, since POLRMT, either alone or together with TFAM or the termination factor MTERF (see below, section 2.2.2), was not able to initiate transcription *in vitro*. Note also the recent finding of Shutt *et al.* (2010), showing that TFAM is not necessary to initiate transcription *in vitro* from LSP or HSP1.

2.2.1.2 Mitochondrial transcription factor A

TFAM belongs to the high mobility group (HMG)–box family of DNA-binding proteins (Parisi and Clayton 1991), and is able to alter mtDNA structure, condensing, unwinding and bending it (Fisher *et al.* 1992) which in turn might facilitate transcription initiation. TFAM protein has two HMG-box domains with a 27 amino acid (aa) linker region between them and a 25 aa C-terminal tail that has been established to be important for accurate DNA recognition, and is limiting for transcriptional activation (Dairaghi *et al.* 1995). Knocking out murine Tfam leads to a decrease in mtDNA copynumber in heterozygous mice and in homozygous mice the knockout is embryonic lethal with massive depletion of mitochondrial DNA (Larsson *et al.* 1998). These findings clearly show that TFAM has an important role in mtDNA maintenance and is also an essential protein for embryonic development (Larsson *et al.* 1998).

TFAM is important in the initiation of mitochondrial transcription, since human mitochondrial RNA polymerase needs TFAM to recognize the promoters of human mitochondrial DNA. TFAM is a rather typical HMG protein in many respects e.g. it prefers binding oxidatively damaged mitochondrial DNA (Yoshida *et al.* 2002), is able to recognize cisplatin damaged DNA where it induces bends (Chow *et al.* 1994, Chow *et al.* 1995). TFAM binds mtDNA showing no sequence specificity (Fisher *et al*. 1989, Fisher *et al.* 1992). The TFAM monomer also binds four-way DNA junctions for which it needs both of the HMG-box domains (Ohno *et al.* 2000). TFAM, like many other HMG-box proteins, can be acetylated: Dinardo *et al*. (2003) reported that TFAM is acetylated at one lysine residue. Ohgaki *et al*. (2007) reported that the C-terminal tail of TFAM strengthens its binding to mtDNA. The evidence presented by Shutt *et al*. (2010) that transcription can be initiated *in vitro* from LSP and HSP1 independently of TFAM, raises questions concerning the primary role of TFAM in mitochondrial transcription.

2.2.1.3 Mitochondrial transcription factor B

A human counterpart of *Saccharomyces cerevisiae* mitochondrial transcription factor B was first reported by McCulloch *et al.* (2002). Human "mtTFB" was shown to bind mtDNA in a non-sequence-specific manner. McCulloch *et al.* (2002) showed that, *in vitro*, mtTFB (now designated TFB1M) and TFAM together are able to activate transcription from the human mitochondrial light-strand promoter. TFB1M can bind S-adenosylmethionine and shows homology to N6 adenine RNA methyltransferases methylating the N6 position of adenine in specific nucleotides in rRNA (McCulloch *et al.* 2002). This was the first report of a transcription factor related to an RNA-modifying enzyme (McCulloch *et al.* 2002).

Falkenberg *et al.* (2002) named two novel ubiquitously expressed transcription factors needed to initiate mammalian mitochondrial transcription as TFB1M and TFB2M: TFB1M is identical to the mtTFB identified by McCullogh *et al.* (2002). Falkenberg *et al.* (2002) used purified recombinant versions of the mitochondrial proteins and suggested that the minimum requirement for transcription from both heavy and light strand human mtDNA promoters consists of a protein complex of TFB1M or TFB2M, TFAM and the mitochondrial RNA polymerase. TFB2M is more active in transcription activation than TFB1M but is also related to bacterial rRNA methyltransferase (Falkenberg *et al*. 2002). Seidel-Rogol *et al.* (2003) reported that TFB1M has two functions: a role in transcription and also as an rRNA methyltransferase, which can methylate a conserved stem-loop both in bacterial 16S rRNA and in the homologous human 12S rRNA molecule. Cotney *et al*. (2007) established, using cultured cells, that TFB2M is primarily the transcription factor, as over-expression of TFB2M induces an approximately 2-fold increase in overall mitochondrial transcript levels whereas TFB1M has no such effect. Using cultured cells over-expressing TFB1M Cotney *et al*. (2007) also first presented *in vivo* evidence that TFB1M is the primary human mitochondrial 12S rRNA methyltransferase. Furthermore, Cotney *et al*. (2009) showed that TFB1M and TFB2M collaborate in mitochondrial biogenesis.

Tfb1m and Tfb2m, the murine TFB1M and TFB2M homologues, are ubiquitously expressed (Rantanen *et al.* 2003). Most metazoans seem to have two TFBM genes (Rantanen *et al*. 2003, Cotney and Shadel 2006). Cotney and Shadel (2006) reported that the two TFBM genes found in metazoans arise from a gene duplication event that took place before the divergence of fungi and metazoans in evolution, and in some organisms the selective pressure finally led to loss one of the genes.

Human TFB1M and TFB2M are both capable of binding the C-terminal tail of TFAM, the region that is needed for the activation of transcription (McCulloch *et al.* 2003). Human TFB1M co-immunoprecipitates with human POLRMT (McCulloch *et al.* 2003) indicating that it forms a link between the human TFAM and POLRMT which would further explain the initiation of transcription in human mtDNA (McCulloch *et al.* 2003). As TFB1M co-immunoprecipitates with POLRMT and *in vitro* has been shown to activate transcription, it is still possible that TFB1M has a role also in transcription which remains to be elucidated.

TFAM is essential in transcription initiation and it is required for POLRMT /TFB2M to be able to recognize the promoter (Gaspari *et al*. 2004). Gaspari *et al.* (2004) proposed that TFAM binds mtDNA inducing a structural change, enabling the POLRMT /TFB2M complex to recognize the promoter sequence. Sologub *et al*. (2009) showed that TFB2M facilitates promoter melting but is not a limiting factor for protein recognition. They also proposed that TFB2M has a role as a transient component of the catalytic site of the transcription initiation complex since it interacts with the priming substrate (Sologub *et al*. 2009). Lodeiro *et al*. (2010) showed using transcription factors A and B2, which were isolated from *Escherichia coli,* that both of them are needed for open complex formation which is the rate-limiting step for production of the first phosphodiester bond whereas the subsequent steps require only TFB2M. Litonin *et al*. (2010) established that only TFAM and TFB2M are needed for successful transcription in *vitro* whereas, as mentioned above, Shutt *et al*. (2010) have presented data indicating that *in vitro* transcription initiation can occur independently of TFAM.

2.2.2 Models for heavy strand transcription

Several models have been proposed to explain the transcription pattern of the heavy strand. Montoya *et al.* (1983) suggested the now generally accepted idea that there are two heavy strand transcription units, one principally for the rRNAs and one for the mRNAs, shown in Figure 2.3. This model was based on the identification of two 5'-triphosphate termini in heavy strand transcripts (Montoya *et al.* 1981, 1982). Transcription starts from two sites, PH1 (16 bp upstream of the $tRNA^{Phe}$ encoding gene) and PH2 (located close to the 5´ end of the 12S rRNA encoding gene) (Montoya *et al.* 1982). Transcription from these sites yields two distinct polycistronic transcripts which are processed to mature transcripts (Clayton 1984). This model also explains why rRNAs are synthesised more frequently than mRNA molecules, as reported by Ojala *et al.* (1981). mRNA and rRNA molecules also have different decay rates which may also affect the relative steady-state levels of these molecules.

According to Montoya's model, transcription unit starting from PH1 is dedicated to transcription of the rRNA genes whereas transcription starting from PH2 produces a primary transcript of almost the whole heavy strand. The protein and rRNA coding genes are flanked by tRNA genes and cutting and processing of tRNAs is a prerequisite to producing mature mRNA and rRNA transcripts. Interestingly, the $tRNA^{Phe}$ gene is read only as a part of the rRNA transcription unit (Montoya *et al.* 1983). On the other hand, $tRNA^{Leu(UUR)}$ is only included in the mRNA transcription unit, since rRNA transcription unit is terminated within the $tRNA^{Leu(UUR)}$ coding sequence.

A competing theory states, based purely on *in vitro* experiments, that there is only one initiation site for heavy strand transcription. According to Ojala *et al*. (1981) there is a premature transcription termination site after the rRNA genes. The two models described above are not completely exclusive and in addition there are several other models which fall somewhere in between them.

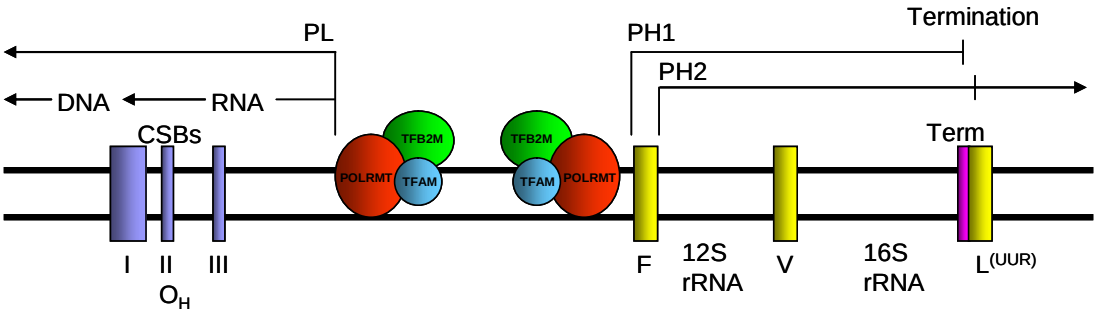

Figure 2.3. Proposed transcription units of the heavy and light strand in mammalian mtDNA and the proteins needed for transcription. The mRNA transcription unit produces a full length transcript of the mtDNA heavy strand. The rRNA transcription unit is dedicated to the transcription of mitochondrial rRNAs and two tRNAs. TFAM is shown here as a part of the transcription machinery although Shutt *et al*. (2010) have recently shown that *in vitro* transcription can occur independently of TFAM from LSP and HSP1. Adapted from Scarpulla (2008).

The finding of two promoter sites *in vivo* strongly indicates that there are two initiation sites for transcription. Both of the models presented above are credible but only one of the promoters has been clearly shown to be functional *in vitro*. These transcription units partially overlap and their initiation sites are located close to each other (Montoya *et al.* 1983). A protein promoting premature termination of transcription immediately after rRNA genes *in vitro* was later characterized, initially supporting the idea of a single initiation site (Kruse *et al.* 1989). This protein, mitochondrial transcription termination factor (MTERF), operates *in vitro* at the gene boundary between 16S rRNA and $tRNA^{Leu(UUR)}$ (Kruse *et al.* 1989). However, Fernandez-Silva *et al.* (1997) showed that, *in vitro*, MTERF alone cannot terminate transcription although *in vivo* studies of this issue have not yet been carried out. Many questions remain to be answered concerning the functions of MTERF. Importantly, even if MTERF turns out to function as a mitochondrial transcription termination factor *in vivo*, this does not exclude Montoya's model.

2.2.3 Mitochondrial light strand transcription

The light strand promoter (LSP) is located near to the origin of the heavy strand replication (see figure 2.3) and therefore it was suggested that LSP also provides heavy-

strand replication with RNA primers. Light-strand transcripts starting from the non-coding region were first found by Wallberg and Clayton (1983) and Chang and Clayton (1985) proved evidence that transcription from LSP, indeed provides RNA primers for replication. Chang and Clayton (1987) furthermore identified and partially purified an mtRNA-processing endoribonuclease (RNase MRP) that is able to cut RNA in a site-specific manner creating the 3'-hydroxyl groups needed for the DNA polymerase to initiate replication. Later, however, mitochondrial heavy strand replication and light strand transcription were showed to be coupled, as the RNA primer for the initiation of mtDNA replication was established to be synthesized in conjunction with transcription and subsequently to remain annealed to the mtDNA template (Lee and Clayton 1998). Recently it has been suggested that very little RNase MRP is found in mitochondria and its role in mtDNA replication is questionable.

2.3 The MTERF protein family

The human MTERF protein was identified and purified some 20 years ago and is the founder member of the MTERF protein family (Linder *et al.* 2005, Chen *et al.* 2005). Human MTERF is the first characterized mitochondrial transcription termination factor based on its *in vitro* activity. The nomenclature of the MTERF proteins, however, is confusing. In this study and thesis the official (HUGO approved) names of the MTERF genes and proteins are used. The nomenclature of the MTERF proteins is shown in Table 2.1. below.

Table 2.1. Nomenclature of the MTERF protein family

Official name	Other names in literature	NCBI accession number (human protein)
MTERF	mTERF, mTERF1	NP_008911
MTERFD1	mTERF3	NP_057026
MTERFD2	mTERF4	NP_872307
MTERFD3	mTERF2	NP_001028222

Vertebrates, other metazoans and plants all have homologues of the human MTERF proteins as reported by Linder *et al.* (2005). The MTERF family proteins share sequence similarity and a conserved 30 aa long MTERF motif. Linder *et al*. (2005) established that there are 4 subfamilies in the MTERF protein family. Vertebrates have all four different MTERF genes and MTERF and MTERFD3 are unique to vertebrates. MTERFD1 and MTERFD2 are found also in worms and insects, and represent the ancestral MTERF genes in metazoans (Linder *et al*. 2005). Interestingly fungi do not contain any MTERF-like proteins which might indicate that fungi lost the MTERF genes early in evolution or else that there has been some kind of lateral gene transfer between ancestral metazoans and plants. In plants, MTERF genes were also observed to have been duplicated many times (Linder *et al*. 2005). All the metazoan MTERF proteins are predicted to be mitochondrial. Most of the plant MTERF proteins are also predicted to be targeted to mitochondria or chloroplasts. Very recently, the MTERF crystal structure was published, shedding more light on the functional role of MTERF and also the other members of the MTERF protein family (Jiménez-Menéndez *et al*. 2010, Yakubovskaya *et al*. 2010).

2.3.1 The mitochondrial transcription termination factor

The human MTERF encoding gene is located on chromosome 7, at locus 7q21-q22 (Fernandez-Silva *et al.* 1997). The mature protein consists of 342 amino acids (Fernandez-Silva *et al.* 1997), having a mitochondrial targeting sequence of 57 amino acids and an alternative start codon located at nucleotide position 138. Daga *et al*. (1993) reported that *in vivo* MTERF exists in 2 or 3 isoforms. The sizes of these isoforms were reported to range from 31 to 34 kDa (Daga *et al*. 1993). A protein corresponding to the 34 kDa sized isoform was shown to terminate transcription *in vitro* (Daga *et al.* 1993). MTERF brings about transcription termination in a biased ‘bipolar’ manner and it is not dependent on POLRMT (Shang and Clayton 1994) (i.e. it works in the presence of other RNA polymerases). The latter authors also were first to report that MTERF is capable of bending mtDNA (Shang and Clayton 1994).

Termination of the rRNA transcription unit occurs at the boundary of the 16S rRNA and $tRNA^{Leu(UUR)}$ genes. MTERF has been proposed to have a limiting role in regulating rRNA synthesis in relation to that other of RNA molecules. Human MTERF specifically is proposed to affect the transcript starting from the rRNA specific initiation site of the heavy strand in the mtDNA (Kruse *et al.* 1989, Daga *et al.* 1993).

Kruse *et al.* (1989) established that MTERF binds a 28 bp sequence in the $tRNA^{Leu(UUR)}$ gene which is downstream and adjacent to the 16S rRNA gene, now also referred to as the canonical binding site. A 13 bp sequence in the middle of this fragment is needed to carry out accurate termination of the rRNA transcription unit i.e. is needed to form the 3' end of 16S rRNA (Christianson and Clayton 1988). The DNA sequence is capable of functioning in a bidirectional manner to bring about accurate termination of transcription (Christianson and Clayton 1986). The A3243G MELAS mutation is situated in the middle of the MTERF DNA binding sequence which makes the functional role of MTERF an interesting topic to study relating to disease. The A3243G MELAS mutation has been shown to decrease the binding affinity of MTERF to its target sequence *in vitro* (Chomyn *et al.* 1992), as well as to downregulate transcription termination *in vitro* (Hess *et al.* 1991). Yakubovskaya *et al*. (2010) furthermore reported that the G3242A mutation prevents MTERF from terminating transcription *in vitro* and that the G3249A mutation prevents MTERF from binding specifically, and therefore decreases MTERF termination activity.

Martin *et al*. (2005) reported a second binding site for MTERF, suggesting that it creates a DNA 'loop' bringing together the transcription initiation and termination sites. They further proposed that this could explain the many fold higher transcription activity of the rRNA genes. Yakubovskaya *et al*. (2010) recently reported that MTERF binds DNA initially in a sequence independent manner which is then followed by sequence recognition. This then leads to an altered DNA structure, unwinding of the DNA double-helix and base flipping. Yakubovskaya *et al*. (2010) also found that MTERF has more interaction sites with the DNA light strand than with the heavy strand which supports the findings of Nam and Kang (2005), who reported that MTERF shows preference for light-

strand binding. These findings might also explain the orientation-influenced termination activity, reported by Asin-Cayuela *et al.* (2005).

The crystal structure of MTERF (Arg56 – Ala399) in complex with the canonical binding site DNA fragment (Jiménez-Menéndez *et al.* 2010, Yakubovskaya *et al*. 2010) shows that the protein comprises nine left-handed helical MTERF repeats, each consisting of three helices. These helices together create a left-handed superhelix called the Zurdo domain, that binds continuous dsDNA altering its structure by inducing a bend in it (Jiménez-Menéndez *et al*. 2010). They also discovered that a shorter variant of MTERF, MTERF ΔN (Arg99 – Ala399), arising due to spontaneous proteolysis, is capable of binding DNA although in a purely nonspecific manner (Jiménez-Menéndez *et al*. 2010). The structural findings refute the suggestion that MTERF contains three leucine zipper motifs forming an intramolecular structure to bring together the two basic domains of MTERF in close proximity to the DNA target sequence (Fernandez-Silva *et al*. 1997).

Prieto-Martin *et al.* (2004a) reported that rat MTERF needs to be in the phosphorylated form in order to carry out efficient transcription termination *in vitro*. Rat MTERF was shown to be phosphorylated at four sites (threonine, tyrosine and serine), but this is not required for binding dsDNA. Asin-Cayuela *et al.* (2005) reported a completely opposite finding, namely that MTERF is functional in transcription termination *in vitro* in the non-phosphorylated form and found no evidence of MTERF being posttranslationally modified. Using recombinant human MTERF, Asin-Cayuela *et al.* (2005) showed that MTERF transcription termination shows clear polarity *in vitro*; when MTERF binds HSP in the forward orientation, transcription is arrested completely, but in the opposite orientation it leads only to a partial arrest of transcription. According to Asin-Cayuela *et al*. (2005) MTERF alone is capable of terminating transcription *in vitro*.

Asin-Cayuela *et al.* (2004) suggested that MTERF is present in mitochondria in two forms, either as an active monomer or as an inactive homotrimer and that MTERF binds

mtDNA and it is active in transcription termination *in vitro* only when it is present as a monomer. Asin-Cayuela *et al.* (2004) also proposed that the quantity of active MTERF in transcription termination is regulated by the transition between the monomer and homotrimer.

2.3.2 MTERF homologues in other organisms

As MTERF is a well conserved protein, its functional role maybe inferred from the properties of its homologues in other organisms. Several MTERF homologues have been cloned and characterized. These include the MTERF homologue in sea urchin, mtDBP (Loguercio Polosa *et al.* 1999), and that from *Drosophila* called DmTTF (Roberti *et al.* 2003). mtDBP and DmTTF are, in terms of evolution, closer to MTERF and to MTERFD3 than to MTERFD1 and MTERFD2 (Roberti *et al*. 2009). The murine homolog of MTERF has also been cloned and characterized (Li *et al.* 2005).

mtDBP is a 348 aa mitochondrially targeted protein binding mtDNA at two distinct sites, in the NCR at the 3´ end of the D-loop and in the gene boundary between the ND5 and ND6 genes (Roberti *et al*. 1991). mtDBP is thought to terminate rather than pause mitochondrial transcription in sea urchins (Fernandez-Silva *et al.* 2001). Loguercio Polosa *et al*. (1999) and Fernandez-Silva *et al*. (2001) showed that mtDBP arrests the mitochondrial RNA polymerase of *Paracentrotus lividus* and that mitochondrial transcription is regulated at the level of transcription termination. mtDBP has been established to be the limiting factor in transcription termination, when the RNA polymerase approaches the mtDBP binding site from the L-strand transcription direction. Interestingly, when RNA polymerase approached from the opposite direction, transcription termination was not affected by mtDBP (Loguercio Polosa *et al*. 2007). Loguercio Polosa *et al*. (2007) therefore suggested that mtDBP is a polar transcription termination factor. mtDBP acts also as a contrahelicase, indicating that it may have a role in DNA replication, possibly as a negative regulator thereof (Loguercio Polosa *et al.* 2005).

DmTTF binds two short non-coding sequences of the *Drosophila* mtDNA that earlier had been predicted to be transcription termination sites (Berthier *et al*. 1986). The binding sites are located in the gene junctions between $tRNA^{Glu}$ and $tRNA^{Phe}$ and between $tRNA^{Ser(UCN)}$ and ND1 respectively (Roberti *et al*. 2003). Roberti *et al*. (2005) established that DmTTF acts as a transcription termination factor *in vitro*, but does not have a role in the formation of the 3´ end of mitochondrial transcripts (Roberti *et al*. 2006b). When DmTTF was knocked down, the transcript levels downstream of the DmTTF binding sites were, however, increased, indicating that knocking down DmTTF removes a transcriptional block (Roberti *et al*. 2006b). Rather surprisingly knocking down DmTTF decreased some transcript levels upstream of its binding sites.

2.3.3 MTERFD1 and MTERFD3

Spåhr *et al*. (2010) have presented the structure of human MTERFD1, which consists of alfa helical tandem repeats displaying a similar triangular three-helix motif as reported for MTERF (Spåhr *et al*. 2010, Jiménez-Menéndez *et al*. 2010, Yakubovskaya *et al*. 2010). Park *et al*. (2007) established that MTERFD1 down-regulates mammalian mitochondrial transcription initiation *in vivo* and that it can bind DNA, preferentially the mtDNA promoter region. In the mouse, homozygous knockout of Mterfd1 is lethal and the mouse embryos die at midgestation (Park *et al*. 2007) indicating that it is an essential gene.

The *Drosophila* homologue of MTERFD1, Mterf3, has been reported to play a role in mitochondrial protein synthesis (Roberti *et al*. 2006a). When Mterf3 was knocked down it did not affect mitochondrial DNA replication, nor did it affect mitochondrial transcription, but altered labelling of mitochondrially translated polypeptides indicates that it plays a role in translation.

MTERFD3 has been reported to be a mitochondrial nucleoid protein (Pellegrini *et al*. 2009). It is expressed at high levels in heart, liver and skeletal muscle (Chen *et al*. 2005) and also, according to Pellegrini *et al*. (2009), it is a rather abundant protein. Pellegrini *et*

al. (2009) estimated that there is one MTERFD3 molecule per 265 bp of mtDNA. MTERFD3 also binds mtDNA in a non-sequence specific manner according to Pellegrini *et al*. (2009). Wenz *et al*. (2009) reported that loss of MTERFD3 leads to decreased levels of mitochondrial mRNA and modulates OXPHOS function in mammals. However, they localized MTERFD3 to the mitochondrial matrix (Wenz *et al*. 2009), and found MTERFD3 to be immunoprecipitated with MTERF and MTERFD1, indicating that these proteins can interact.

2.4 Mitochondrial translation

Barrell *et al*. (1979) first reported that the mammalian mtDNA genetic code differs from the so called universal code. Four out of the 64 codons have a different meaning in the mammalian mitochondrial genome when compared to other genomes. The unique features of the mammalian mitochondrial genetic code are listed in Table 2.2. In human mitochondria the ARG triplets as listed in Table 2.2. appear to serve as termination signals, which is seen rarely in other mammals. Temperley *et al*. (2010) showed that in human mitochondria these ARG triplets, likely together with other *cis* elements, promote frameshifting in the mitoribosomes.

Table 2.2. Differences between the universal code and the mammalian mitochondrial genetic code.

Codon	Universal genetic code	Mitochondrial genetic code (mammals)
UGA	STOP	Trp
AUA	Ile	Met
AGA	Arg	STOP
AGG	Arg	STOP

Nuclear-encoded factors are needed for mammalian mitochondrial translation, as reviewed by Rorbach *et al*. (2007). Protein synthesis in the cytosol (or in chloroplasts in

plants) requires 30 or more different tRNAs, whereas only 22 different tRNAs are needed for human mitochondrial protein synthesis (Anderson *et al.* 1981). In mitochondria many tRNA molecules can recognize any of the four nucleotides in the third ”wobble” position in the triplet because of the relaxed codon-anticodon pairing rule (Barrell *et al.* 1980). Mitochondrial amino acids generally need only one tRNA to read their codons but there are two exceptions; leucine and serine require two tRNAs each. $tRNA^{Leu(UUR)}$ and $tRNA^{Leu(CUN)}$, and $tRNA^{Ser(AGY)}$ and $tRNA^{Ser(UCN)}$ are dedicated to separate codon groups.

2.5 The principles of DNA replication

Conventionally, DNA is copied semi-conservatively i.e. replicated so that each of the parental strands is used as a template (see Figure 2.4) to produce the complementary strands as reviewed in Alberts *et al*. (2002). Only as the replication event is over and the nascent DNA strands -consisting of one parental strand and its complementary strand- are ready, they are separated.

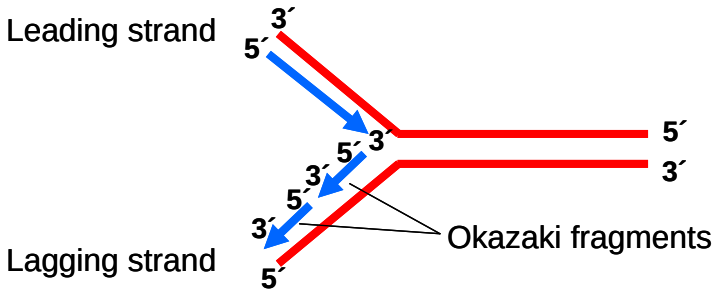

Figure 2.4. DNA replication fork. DNA polymerase synthesizes DNA in the 5´ to 3´ direction and therefore the lagging strand has to be replicated in Okazaki fragments.

The protein machinery required for DNA replication consists of several proteins having different roles in the replication process as illustrated in Figure 2.5. DNA polymerase catalyzes DNA synthesis, for which it requires a primer (a short RNA or DNA fragment). DNA polymerase synthesizes DNA in the 5´ to 3´ direction. Before DNA polymerase can advance, the DNA double helix needs to be opened. This is done by a protein called DNA

helicase. It opens up the hydrogen bonds between the parental DNA strands, the unwinding leading to supercoiling of the double stranded DNA template and creating torsional stress. A protein called topoisomerase is thus needed to remove torsional stress and supercoiling. Therefore, both helicase and topoisomerase are crucial for the DNA polymerase to be able to copy the template DNA.

There are a few additional proteins needed for successful DNA replication. As the lagging-strand is synthesized discontinuously, as Okazaki fragments, the parental strand is temporarily left exposed. Single-strand binding protein SSB is needed to protect the exposed single strand before the synthesis of the lagging strand takes place. Synthesis of the lagging strand also needs a primer, and the priming is carried out separately for each advancing Okazaki fragment to be elongated by the DNA polymerase. Once the Okazaki fragment reaches the primer sequence of the previously completed Okazaki fragment, the primer is removed by a ribonuclease, and DNA synthesis is continued up to the first nucleotide of the preceding fragment. Finally a DNA ligase is needed to join together the Okazaki fragments to create a continuous DNA strand.

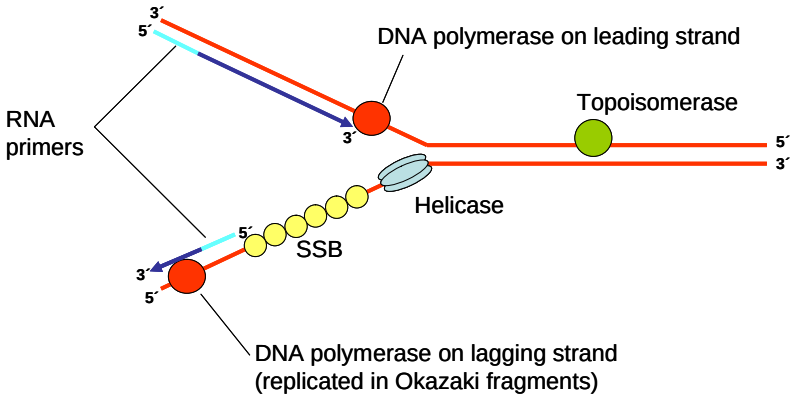

Figure 2.5. The protein machinery required for DNA replication.

2.6 Replication of human mtDNA

2.6.1 Different models of replication

As the mitochondria originate from the symbiotic relationship of an early eukaryotic cell with a prokaryotic cell, the interesting question is whether the replication of the modern-day mitochondrial DNA resembles more that of its prokaryotic ancestor cell or that of eukaryotic cells. There are two competing models to describe mtDNA replication, the strand-displacement model and the conventional, coupled leading- and lagging-strand DNA synthesis model.

2.6.1.1 Strand-asynchronous replication of mtDNA

In 1972, studies by electron microscopy indicated that mammalian mtDNA is replicated in an unconventional way, the replicating mtDNA having a single-stranded branch (Robberson *et al*. 1972). This gave rise to the model presented by Clayton (1982), referred to as the strand-displacement model involving strand-asynchronous and asymmetric DNA replication. Until quite recently, mtDNA replication was thought to occur uniquely as described in this model.

In the strand-displacement model the mtDNA strands are replicated at different times as shown in Figures 2.6 and 2.8a. According to this model there are two replication initiation sites for the two strands, heavy (leading) and light (lagging) strands, and these initiation sites are located far apart from each other in the circular genome (Clayton 1982). Replication starts from the heavy strand replication (illustrated as green dotted line in Figure 2.6) initiation site, O_H, and two thirds of the leading strand is replicated before replication of the lagging strand from O_L (illustrated as red dotted line in Figure 2.6), the light strand replication initiation site, in the opposite direction is initiated. As the heavy strand is replicated the parental strands are separated from each other. Once the initiation site for light strand replication has been uncovered, lagging strand replication may begin.

All this involves continuous ssDNA synthesis taking places in both directions (Clayton 1982).

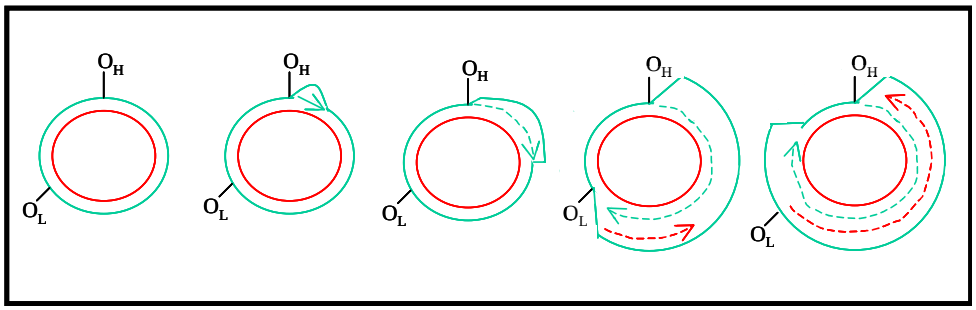

Figure 2.6. The strand-asynchronous replication model of the human mitochondrial DNA. Adapted from Clayton (2000).

2.6.1.2 Strand-synchronous replication of mtDNA

Holt *et al*. (2000) first presented evidence for a more conventional mode of mtDNA replication based on coupled leading- and lagging-strand DNA synthesis (see Figure 2.8b). This mechanism is similar to the one used to replicate mammalian nuclear DNA (Lodish *et al.* 1999). This model suggests that the two strands are replicated symmetrically and it occurs simultaneously. Only one replication initiation site is therefore needed and replication proceeds on both strands in the same direction at the same time. Bowmaker *et al*. (2003) reported that bidirectional replication starts downstream of O_H that is the heavy strand replication origin. Yasukawa *et al*. (2005) mapped two sites in the NCR for bidirectional replication initiation in cultured cells recovering from drug-induced mtDNA depletion. At O_H one of the replication forks is stalled whereas the other one continues replicating through the whole genome as shown below in Figure 2.7. The replication machinery replicates mtDNA only in the conventional 5' - 3' direction meaning that one of the strands is replicated in Okazaki fragments (see Figure 2.8b). Black arrows in Figure 2.7 denote the replication fork movement direction.

Holt *et al.* (2000) suggested that these two replication mechanisms might function in mammalian mtDNA under different conditions. Namely, the two replication mechanisms

introduced above are not mutually exclusive. In support of this idea, it was found that strand-coupled replication occurs after EtBr-induced mtDNA depletion when cells replicate mtDNA more actively. When the mtDNA is only maintained in cells that grow 'normally' replication seems to occur according to a mechanism more akin to the model presented by Clayton (1982). Twenty-four hours after mtDNA depletion, cells were found to contain replication intermediates from both replication modes indicating that the cells were shifting back to normal mtDNA maintenance from active amplification mode. Therefore, it is also possible to hypothesize that Clayton's model with its single light strand replication initiation site is an extreme case of Holt's model (Holt *et al.* 2000).

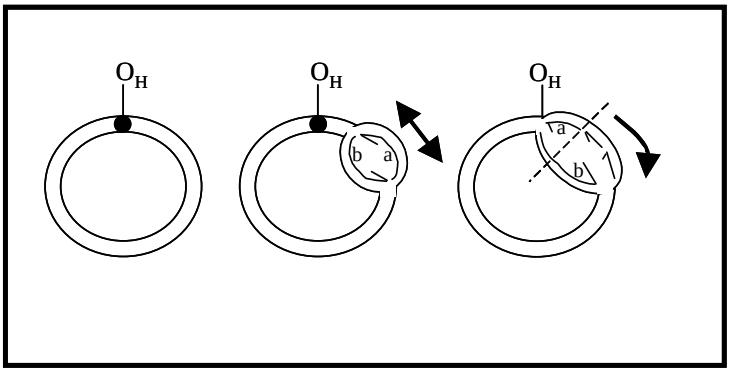

Figure 2.7. Bidirectional strand-coupled replication of mtDNA. Adapted from Bowmaker *et al*. (2003) and Yasukawa *et al*. (2005).

2.6.1.3 RITOLS replication

There are many recent publications where authors using 2DNAGE have failed to detect the single stranded replication intermediates predicted by the Clayton model (Holt *et al*. 2000, Kajander *et al.* 2001, Yang *et al*. 2002, Bowmaker *et al*. 2003, Reyes *et al*. 2005, Yasukawa *et al*. 2005, Yasukawa *et al*. 2006). Instead of such partially single-stranded replication intermediates, two groups of double stranded replication intermediates were reported in these publications. The first are intermediates originating from coupled leading- and lagging-strand DNA synthesis (Holt *et al*. 2000, Kajander *et al*. 2001, Reyes *et al*. 2005, Yasukawa *et al*. 2006). Secondly, Yang *et al*. (2002) established that the mammalian mtDNA contains extended regions of RNA:DNA hybrid as ribonucleotides,

incorporated on the light strand, which are finally converted to DNA (see Figure 2.8c). Yasukawa *et al*. (2006) subsequently found replication intermediates containing long RNA segments extending over the whole lagging-strand before lagging-strand DNA synthesis had taken place, introducing the concept of RITOLS replication i.e. RNA incorporation throughout the lagging strand.

These two groups of replication intermediates not only have different ribonucleotide content but also their initiation sites differ. Coupled leading- and lagging-strand DNA synthesis initiates from a broad zone of many kilobases (Bowmaker *et al*. 2003, Reyes *et al*. 2005), whereas RITOLS replication initiates only within the NCR (Yasukawa *et al*. 2006). This finding indicates that replication might occur in several different ways in vertebrate mtDNA. On the other hand, the RITOLS type of replication resembles the strand-asynchronous model presented by Clayton (1982), since there is considerable delay between leading and lagging-strand DNA synthesis in both models. The single stranded replication intermediates visualized by means of electron microscopy in 1970´s and later by means of atomic force microscopy (Robberson *et al*. 1972, Kasamatsu and Vinograd 1973, Brown *et al*. 2005) may be explained on the basis of loss of ribonucleotide segments from RITOLS RIs.

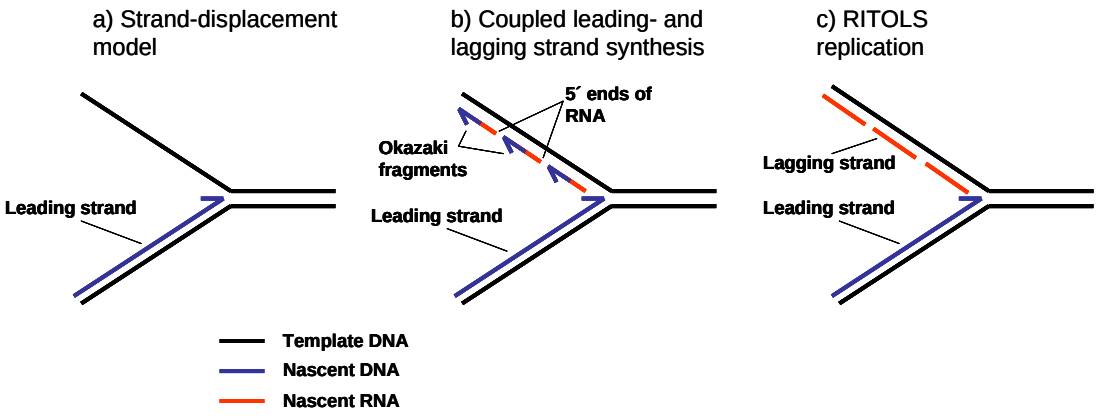

Figure 2.8. Different modes of mtDNA replication. a) Strand-displacement model. b) Conventional coupled leading- and lagging-strand DNA synthesis. c) RITOLS replication. (Adapted from Holt 2009).

2.6.2 mtDNA replication machinery

2.6.2.1 mtDNA Polymerase gamma

The genes encoding components of the mtDNA replication machinery have been cloned and the proteins characterized only rather recently, compared to other systems. So far the only DNA polymerase implicated in mtDNA replication is the mtDNA polymerase gamma (POLγ) (Gray and Wong 1992, Ropp and Copeland 1996, Lecrenier *et al.* 1997). Walker *et al*. (1997) mapped the human POLγ coding gene to chromosome 15 at locus 15q25. It resembles both the *E. coli* DNA polymerase I and T7 DNA polymerase, belonging to the family A group of DNA polymerases (Fridlender and Weissbach 1971, Fridlender *et al*. 1972, Beese *et al*. 1993). The POLγA (catalytic) subunit polymerizes DNA in the 5´→3´ direction and also has proofreading exonuclease activity acting in the 3´→5´ direction (Gray and Wong 1992). POLγA synthesizes DNA at 180 bp/min rate according to Korhonen *et al*. (2004).

POLγA interacts with the mtDNA polymerase γ subunit, POLγB (Gray and Wong 1992), the structure of which highly resembles class IIa aminoacyl tRNA synthetases (Fan *et al*. 2006). POLγB binds double stranded DNA of at least 45 bp, and also forms a functional homodimer (Carrodeguas *et al.* 2001). Carrodeguas *et al.* (2002) established that both DNA binding sites are needed for binding DNA since β subunit heterodimers, in which one of the two DNA binding sites is mutated, could not bind dsDNA. Together with POLγA, POLγB forms a heterotrimer, $POL\gamma AB_2$ (Yakubovskaya *et al*. 2006). POLγB prevents DNA polymerase gamma from falling off when it finds a local difficulty in the template DNA strand, notably enhances the processivity and the catalytic effectiveness of the enzyme, and is needed to maintain the structure of the holoenzyme (Carrodeguas *et al.* 1999, Lim *et al.* 1999). Fan *et al*. (1999) suggested that POLγB recognizes the primer that is needed for replication initiation.

When considering the *Drosophila* replication machinery, Wernette and Kaguni (1986) purified the mitochondrial DNA polymerase from *Drosophila* embryos and found two

proteins, one of 125 kDa and other of 35 kDa, in 1:1 ratio, and concluded that the *Drosophila* DNA polymerase gamma is a heterodimer. Kaguni and Olson (1989) detected 3´ → 5´ exonuclease activity associated with the mitochondrial DNA polymerase γ in *Drosophila* embryos. Lewis *et al*. (1996) cloned a cDNA encoding the catalytic subunit. The implied molecular mass of the protein was 129.9 kDa. The catalytic subunit shows a high degree of amino acid sequence conservation to *Escherichia coli* DNA polymerase I (Klenow fragment) (Lewis *et al*. 1996). Wang *et al*. (1997) cloned and sequenced a cDNA encoding the accessory subunit in from *Drosophila* embryos. The predicted size of the protein was 41 kDa. Iyengar *et al*. (2002) provided evidence that the accessory subunit is required for replication and for development. Luo and Kaguni (2005) studied the functional role of the spacer region between the exonuclease and DNA polymerase domains of the catalytic subunit and showed that mutating conserved sequence elements therein led to changes in enzyme activity, processivity and/or DNA binding affinity in three cases out of four. Mutations were also found to affect interaction with the mtSSB (Luo and Kaguni 2005). The conserved sequence elements in the spacer region were suggested to serve to place the substrate in the correct orientation with respect to the polymerase catalytic domains (Luo and Kaguni 2005).

2.6.2.2 Mitochondrial transcription factor A in mtDNA replication

TFAM has been characterized as an essential protein in bringing about transcription initiation *in vitro* at the HSP and LSP promoter sequences, together with the mitochondrial RNA polymerase and TFB1M or TFB2M (Fisher and Clayton 1988, Falkenberg *et al*. 2002), although Shutt *et al*. (2010) have questioned the role of TFAM in this process. Light strand transcription potentially provides the primer for leading-strand synthesis, which is why TFAM has been suggested to have an important role in mtDNA replication. TFAM is also a mitochondrial nucleoid protein (Alam *et al.* 2003, Garrido *et al*. 2003), see also above in section 2.1.6.

2.6.2.3 Mitochondrial single-strand DNA-binding protein

Pavco and van Tuyle (1985) reported the characterization of a rat mitochondrial protein needed to stabilize single-stranded DNA fragments during replication or repair, mitochondrial single-strand DNA binding protein (mtSSB). Tiranti *et al.* (1993) cloned the human and rat mtSSB encoding cDNAs. Thömmes *et al.* (1995) isolated the *Drosophila* mtSSB of 18 kDa from *Drosophila* embryos. *Drosophila* mtSSB is capable of binding a ~17 nt fragment of mtDNA, and it upregulates replication initiation (Thömmes *et al.* 1995). Farr *et al.* (1999) published that *Drosophila* mtSSB enhances both the 5´ → 3´ DNA polymerase and 3´ → 5´ exonuclease activities of *Drosophila* DNA polymerase γ by 15-20 fold and stimulates the initiation of DNA strands 30-fold. Maier *et al.* (2001) showed that the *Drosophila* mtSSB mutation *lopo*^{1} leads to mtDNA depletion and loss of respiration. The *lopo*^{1} mutants die before metamorphosis is complete. Maier *et al.* (2001) showed that *Drosophila* mtSSB is essential both for mtDNA replication and development. Farr *et al.* (2004) established that mtSSB mutants defective for DNA binding cannot stimulate DNA replication by pol γ.

The human mtSSB is a protein of about 16 kDa and its sequence resembles that of *E. coli* SSB (Curth *et al.* 1994). It forms a tetramer in solution and binds 50-70 nt per tetramer (Curth *et al.* 1994). It has been shown to function also in the resolution of mtDNA junctions by enhancing RecG, which is a junction-specific helicase from *E. coli* (Takamatsu *et al.* 2002). mtSSB and TFAM have been suggested to stabilize the D-loop region together (Takamatsu *et al.* 2002). mtSSB has been also reported specifically to stimulate Twinkle protein activity (Korhonen *et al.* 2003).

2.6.2.4 Mitochondrial primase

In both replication mechanisms there also must exist a primase that makes at least a short RNA-primer. This primer is supplied by transcription, hence replication and transcription are coupled processes in mitochondria. Until quite recently the mechanism of lagging strand replication initiation has remained unclear: although Wong and Clayton (1985)

reported primase activity in human mitochondria but never identified the protein. Twinkle helicase has been a candidate for human mitochondrial primase as it has primase activity in some nonmetazoan cells (Shutt and Gray 2006). However, it has became clear that the human mitochondrial Twinkle protein has lost this activity (Farge *et al*. 2008). Wanrooij *et al*. (2008) reported that mitochondrial RNA polymerase (POLRMT) shows a primase activity *in vitro*. Fuste *et al*. (2010) finally established that the POLRMT has a primase activity which could potentially serve for the initiation of lagging-strand synthesis on longer, double stranded DNA templates. Fuste *et al*. (2010) showed that depleting POLRMT causes decreased replication initiation at the lagging strand replication initiation site *in vivo*. On the basis of *in vitro* studies they suggest that leading-DNA synthesis starts from the O_H, continues till O_L and when O_L is exposed, adopts a stem-loop structure. POLRMT then initiates primer synthesis from a poly-dT sequence in the single-stranded loop, after which it then synthesises about 25 nt until it is replaced by POLγ. However, proof that this is the mechanism whereby the lagging strand initiates *in vivo* is still lacking.

2.6.2.5 Mitochondrial DNA helicase, Twinkle

The Twinkle sequence highly resembles that of phage T7 gene 4 primase/helicase (Spelbrink *et al.* 2001), and belongs to the RecA/DnaB superfamily. Ziebarth *et al*. (2007) showed that the human mtDNA helicase belongs to the DnaB-like family of helicases. Characteristically for protein functioning in mtDNA metabolism Twinkle co-localises with mtDNA and is also a nucleoid protein. *In vitro* functional Twinkle protein forms hexamers in solution just like other ring helicases (Spelbrink *et al.* 2001). Ziebarth *et al*. (2007) established, using velocity-sedimentation and gel-filtration analyses, that the human mtDNA helicase indeed forms hexamers. They established that the human mtDNA helicase and bacteriophage T7 primase-helicase show similar modular architecture and that both proteins form hexamers.

Korhonen *et al.* (2003) established that Twinkle indeed has 5'→3' helicase activity. It opens the DNA double strand for the proceeding DNA polymerase and therefore creates

torsional stress and supercoiling in the DNA duplex. Both groups failed to find primase activity for Twinkle. Matsushima and Kaguni (2007) cloned the *Drosophila* counterpart of the human mtDNA helicase, Twinkle. They showed that silencing *d*-mtDNA helicase leads to a decrease in mtDNA copynumber whereas over-expression leads to an increase. Furthermore Matsushima and Kaguni (2009) failed to detect any primase activity of the *Drosophila* mtDNA helicase. Certain N-terminal amino acid residues, mapping in the vicinity of the central linker region, have been suggested to be important for the C-terminal helicase activity (Matsushima and Kaguni 2009).

The human Twinkle needs mitochondrial ssDNA binding protein to be efficient in unwinding DNA (Korhonen *et al.* 2003). Twinkle has been reported to be mutated in some patients affected with progressive external ophtalmoplegia (PEO) (Spelbrink *et al.* 2001). Dominant Twinkle mutations cause accumulation of mtDNA replication intermediates in cultured cells and also mtDNA depletion (Goffart *et al*. 2009). Goffart *et al*. (2009) suggested that Twinkle PEO mutations lead to mtDNA replication stalling and that this finally leads to multiple deletions characteristic of PEO patient mtDNA.

2.6.2.6 Other proteins needed in mitochondrial replication

Other proteins are also needed for mitochondrial replication. A topoisomerase is needed to remove the torsional stress and supercoiling created by the replicative helicase. Zhang *et al.* (2001) cloned the mitochondrial topoisomerase, Top1mt, which has a mitochondrial targeting sequence and shows homology to the nuclear topoisomerase, Top1. They also showed that when Top1mt was inhibited by camptothecin 7S DNA levels were reduced, indicating that Top1mt might have other, as yet unrecognized roles in mtDNA maintenance (Zhang and Pommier 2008).

Lakshmipathy and Campbell (1999) identified a mitochondrial DNA ligase that is potentially the one needed in both replication models to seal new strands to form circles and also in the strand-synchronous model to join together the Okazaki fragments. Finally, mitochondrially localized RNaseH1, which should be needed for removing short RNA

primers that arise during lagging-strand priming, has been reported to be able to process also long RNA/DNA hybrids (Gaidamakov *et al*. 2005).

2.6.3 mtDNA replication pausing

Controlled replication and/or transcription termination and pausing are of outmost importance. For example, in bacteria and yeast unregulated head-on collision of two protein machineries can inhibit DNA replication, therefore creating genomic instability (Bierne and Michel 1994, Weitao *et al*. 2003, Mirkin and Mirkin 2005, Prado and Aguilera 2005).

In mammalian cell nuclei such head-on collision of two protein machineries can cause dramatic gene amplification events (Hashizume and Shimizu 2007). Having an important role in maintaining genomic stability, the pause binding proteins constitute an interesting object for study.

Tus protein in *E. coli* regulates chromosomal DNA replication termination, providing a good example of a protein with contrahelicase activity (Mulcair *et al*. 2006). *Ter* sequences, which are Tus binding sites, are scattered around in the terminator region. Tus binds *Ter* and therefore stops the advancing replication fork in a directional manner at this terminator region (Hill *et al*. 1989, Neylon *et al*. 2005). Mitochondrial replication pauses have been reported for mtDNA as well, but no Tus homologue has yet been found (Mayhook *et al*. 1992, Holt *et al*. 2000, Reyes *et al*. 2005). Tus protein, however, is not only able to stall an advancing replication fork but is also able to block transcription (Mohanty *et al*. 1996). In fact, Tus prefers to block transcription in the same polar fashion, permitting DNA synthesis to proceed (Mohanty *et al*. 1996, Guajardo and Sousa 1999). The sea urchin MTERF homologue mtDBP also has contrahelicase activity (Loguercio Polosa *et al*. 2005). So far mtDBP is the only mitochondrial protein with characterized contrahelicase activity *in vitro* (Loguercio Polosa *et al*. 2005). It remains to be established which, if any, of the previously characterised human mitochondrial

proteins has contrahelicase activity or whether there remains a novel mitochondrial protein awaiting discovery.

3. Aims of the research

The main purpose of this work is to further characterize the functional role of the human MTERF family of proteins in mitochondrial transcription and replication. Also the role of TFAM in human mitochondrial DNA metabolism is further elucidated.

More specifically the aims of the work were as follows:

1) To further elucidate the role of MTERF in mtDNA transcription termination *in vivo*: MTERF has been previously shown to promote premature heavy strand transcription termination *in vitro* at the canonical binding site following the rRNA encoding genes in human mitochondrial DNA.

2) To determine if MTERF has any novel yet unknown binding site(s) in mtDNA in addition to its canonical binding site within the $tRNA^{Leu(UUR)}$ coding gene. This might shed further light on its possible role(s) in mtDNA metabolism.

3) To test whether MTERF has a role in human mtDNA replication, as well as its putative role in transcription.

4) To determine whether the novel MTERF protein family members, MTERFD1 and MTERFD3 show sequence-specific DNA binding activity.

5) To investigate whether MTERFD1 and/or MTERFD3 have a role in human mtDNA replication.

6) To test whether modulating TFAM levels in cultured human cells affects mitochondrial tRNA transcript production; as a by-product of this work, conducted in collaboration with J. Pohjoismäki, it was additionally of interest to compare the effects of TFAM and MTERF modulation on mtDNA replication.

4. Materials and methods

4.1 Plasmid DNA constructs and molecular cloning

4.1.1 MTERF constructs

The MTERF coding region containing the mitochondrial targeting signal and the protein coding sequence, a total of 1197 bp starting from the first start codon, was amplified from HeLa cDNA. cDNA had been prepared by reverse transcription of 0.8 μg of HeLa mRNA (cDNA was a gift from Professor Johannes Spelbrink). The MTERF coding region was amplified using cloned *Pfu* polymerase (Stratagene) and chimeric primers. MTERF was amplified with and without its natural STOP codon. A C-terminal fusion with the MycHis epitope tag was created using the following primers, BamHI-MTERF F1 5´ - CGCGGATCCCTGTTCTCCAGCCTTTCTGG - 3´ together with HindIII-MTERF R1 5´ - CCCAAGCTTGGCAAATCTGCTTAACTTTTTC - 3´ and an MTERF expression construct with the natural stop codon was created using the following primers, BamHI-MTERF F1 plus HindIII-MTERF R STOP 5´ - CCCAAGCTT*TCA*GGCAAATCTGCTTAACTTTTTC - 3´. Here restriction sites are underlined and the stop codon is shown in italics. Constructs were sequence verified, digested with *Bam*HI and *Hind*III (Fermentas) and ligated using T4 DNA ligase (Fermentas) with pcDNA3.1(-)/Myc-His A (Invitrogen) or pcDNA3/hygro(-) vector (Invitrogen) vector that was similarly digested. Inducible Flp-In™ T-Rex™-293 cell lines expressing MTERF and MTERF-MycHis were created subsequently by digesting the previously created plasmid constructs with *Pme*I (New England Biolabs) and ligating to similarly digested pcDNA5/FRT/TO vector (Invitrogen).

4.1.2 MTERFD1 and MTERFD3 constructs

The MTERFD1 (CGI12) coding region, in total 1254 bp, was amplified from HeLa cell cDNA using the following chimeric primer pair: KpnI/Terfin1F1 5´ - AAAGGTACCACGGGAAGCAGGCCTCGCCACAGACTAAG – 3´ plus XhoI/Terfin1flagR1 5´ - TTTCTCGAGCTTATCGTCGTCATCCTTGTAATCAAGCG TTTTTAAGAA – 3´ creating an in-frame C-terminal fusion to the FLAG epitope tag. In order to create an MTERFD1 expression construct with natural stop codon cDNA was amplified with KpnI/Terfin1F2 5´ - CGCGGTACCAGGCCTCGCCACAGACTAAG – 3´ and XhoI/Terfin1stopR2 5´ - GCCCCTCGAG*TCA*AAGCGTTTTTAAGAA TTTTTCAAA – 3´. Restriction sites are underlined and the stop codon is shown in italics.

Similarly the MTERFD3 (LOC80298) coding sequence of 1158 bp was cloned to create an in-frame C-terminal fusion to the HA epitope tag using chimeric primer pair KpnI/Terfin2F1 5´ - GGGGGTACCCCCAGGACGGTGGAAACTAGCTAGTAGATT GC – 3´ plus XhoI/Terfin2HAR1 5´ - TTCTCGAGAGCGTAATCCGGAACATCGTA TGGGTATTCTTCAACATTTAA – 3´. To create an MTERFD3 expression construct with natural stop codon, the following chimeric primers were used: KpnI/Terfin2F2 5´ – CCCGGTACCGTGGAAACTAGCTAGTAGATTGC – 3´ and XhoI/Terfin2stopR2 5´ - CCCCTCGAG*TCA*TTCTTCAACATTTAATGG –3´. In addition, MTERFD3 was cloned using the following chimeric primers to produce an in-frame C-terminal fusion to the FLAG epitope tag, KpnI/Terfin2F2 5´ – CCCGGTACCGTGGAAACTAGCTAGTAGATTGC - 3´ plus XhoI/Terfin2flagR2: 5´ – TTTCTCGAG*TCA*CTTATCGTCGTCATCCTTGTAATCTTCTTCAACATTTA – 3´. Restriction sites are underlined and the stop codon is shown in italics.

After the sequences were verified, PCR products were digested with *Xho*I and *Kpn*I (New England Biolabs), ligated to similarly digested pcDNA3/hygro(+) vector (Invitrogen) or pcDNA5/FRT/TO (Invitrogen) and stably transfected into Flp-In™ T-REx™ 293 recipient cells (Invitrogen) as described by Wanrooij *et al*. (2007). FLAG

epitope-tagged MTERFD3 expression construct was cloned only into the pcDNA3/hygro(+) vector.

4.1.3 TFAM constructs

The TFAM gene was amplified from a cDNA clone (Maniura-Weber *et al*. 2004) as described in detail in paper IV.

4.2 Bacterial and mammalian cell culture

4.2.1 Bacteria

Bacteria used in this study were chemically competent *E. coli* cells of XL1-blue strain (Stratagene) which were cultured in LB-medium (1% tryptone (Pronadisa), 0.5% Yeast extract (Pronadisa), 0.5% NaCl) containing 0.1 mg/ml of ampicillin (Sigma) or 0.05 mg/ml kanamycin (Sigma) for selection.

4.2.2 Cell culture

Human embryonic kidney cells line, HEK293T, 143B osteosarcoma cells (and cybrids containing wild type mtDNA), HeLa, Jurkat and Flp-In™ T-Rex™-293 cells (Invitrogen) were cultured at 37 °C in an incubator with 5% CO_2 in air in Dulbecco's modified Eagle's medium (DMEM, Sigma) with the following additives: 4.5 g/l of D-glucose, 10% foetal calf serum (FCS) (GibcoBRL), 50 μg/ml uridine (Sigma) and 2 mM L-Glutamine (Bio-Whittaker/Cambrex). Flp-In™ T-Rex™-293-derived cell lines were blasticidin and hygromycin selected in culture according to the manufacturer's protocol, induced to express the protein of interest with 10 ng/ml doxycyclin (Sigma-Aldrich) which was renewed every 48 h. Stably transfected HEK293T cells were cultured under 2 mg/ml G418 Sulfate (Calbiochem) or hygromycin (Calbiochem, 200 μg/ml) selection. Cells

were routinely passaged every 3-4 days at 1:10-1:20 dilution. HeLa cells were detached with Trypsin-EDTA (Bio-Whittaker/Cambrex) and other adherent cells by pipetting only. Jurkat cells growing in suspension were passaged by centrifugation and resuspension in fresh medium.

4.3 DNA transfections

Plasmid DNA constructs were introduced into HEK293T cells by transfection of 3 μg of plasmid DNA and using 30 μl of LipofectAMINE transfection reagent (Invitrogen, protocol supplied by the manufacturer) or 10 μg of DNA and 40 μl of TransFectin™ Lipid Reagent (Bio-Rad, protocol supplied by the manufacturer). Transiently transfected cells were either harvested for different assays typically 24 h or 48 h after transfection, or placed under selection using 2 mg/ml G418 Sulfate (Calbiochem) or hygromycin (Calbiochem, 200 μg/ml) in order to select stably transfected clones.

4.4 Establishing Flp-In™ T-Rex™-293 cell lines

Flp-In™ T-REx™ 293 cell lines were created as described by Wanrooij *et al*. (2007).

4.5 Doubly expressing cell lines

In order to create cell clones expressing two proteins of interest simultaneously, uninduced Flp-In™ T-Rex™-293 cells previously transfected with either MTERFD1-FLAG or MTERFD3-HA were transfected a second time with an MTERF-MycHis expression construct with G418 resistance coding gene. Doubly expressing clones were screened by means of Western blotting.

4.6 DNA sequencing

Expression vector constructs and DNA probes were sequence-verified using the ABI PRISM® BigDye™ Terminator Ready Reaction kit (AppliedBiosystems). Primers that were used for sequencing were either vector specific M13F, M13R, T7, BGH or transgene specific. Sequencing reactions were ethanol precipitated and resuspended in HiDi Formamide (AppliedBiosystems). Resuspended DNA products were denaturated at 92°C for 2 min and subsequently cooled on ice. An ABI310 or ABI3100 Genetic Analyzer (AppliedBiosystems) was used for separation and analysis of the sequencing PCR products under denaturing conditions, according to manufacturer's protocol.

4.7 RNA interference

4.7.1 RNAi constructs

4.7.1.1 siRNA constructs

A prediction programme (http://www.ambion.com/techlib/misc/siRNA_finder.html) was used to design the MTERF specific siRNAs, which were synthesized by means of *in vitro* transcription using the Silencer™ siRNA construction kit (Ambion). Five siRNAs were tested and one was found by Western blotting to be efficient in MTERF silencing and that one was chosen for further experiments. The target site in MTERF mRNA was nt 585–605 (5′ - AAG CGG GUG AAA GCU AAC AUU - 3′).

4.7.1.2 Lentiviral shRNA vector constructs

shRNAmir inserts (Open Biosystems Expression ArrestTM pSM2 Retroviral shRNAmir Library (Thermo Fisher Scientific, Huntsville, AL, USA)) were delivered in pSM2c retroviral vector (Open Biosystems) with one exception. Insert containing constructs were digested with *Xho*I (NEB) and *Mlu*I (NEB) and religated in similarly digested

pGIPZ vector (Open Biosystems). The constructs found to be efficient in silencing based on Western blotting were the following: for MTERF (5´ to 3´): GCUGUAACUUGAGUACUUU, Oligo ID V2HS_95064; for MTERFD1 (5´ to 3´): CCUCAGAUAUCCUCUGACA, Oligo ID V2HS_235060; for MTERFD3 (5´ to 3´): CAGGCUGUGUUCUUUCAGA, Oligo ID V2HS_137356 when compared to cells transfected with Empty pGIPZ vector (Open Biosystems). In addition, the following specific shRNA constructs, all in 5´ to 3´ orientation, were also tested for MTERFD1, GCUGUUUAAGGUCAAAGAA, Oligo ID V2HS_236922 (denoted shRNA 4 in Supplementary Figure S3 of III); for MTERFD3, CGCUGUUAACACCCAGAGAA, Oligo ID V2LHS_236692 (supplied in vector pGIPZ) (denoted shRNA 8 in Supplementary Figure S3 of III) and for MTERF, CUGUAACUUGAGUACUUUA, Oligo ID V2HS_231874 (denoted shRNA 5 in Supplementary Figure S3E of II).

4.7.2 RNAi transfections

MTERF was knocked down in HEK293T cells (either with or without prior stable transfection with the MTERF-MycHis expression construct) by transfecting the cells with 10 nM (final concentration) MTERF-specific siRNA molecules using Lipofectamine™ 2000 transfection reagent (Invitrogen, protocol supplied by the manufacturer). An siRNA reagent targeted on 5′- GGA GAA GGU ACG AGG GGC AUU -3′ (siRNA Control) was used as a negative control. Cells were harvested 48 h after transfection with the siRNA or, in the case of the depletion assay with EtBr, at different time points following the EtBr treatment.

When transfecting cells with shRNAmir constructs, 10 μg of plasmid construct DNA was introduced to cells using Lipofectamine™ 2000 (Invitrogen) transfection reagent. Cells were harvested 48 h after transfection.

4.8 SDS-PAGE and Western blotting

Proteins were extracted as previously described by Spelbrink *et al*. (2000). Protein concentrations were determined according to Bradford (1976). SDS-PAGE using 12% polyacrylamide gels was run under standard conditions (Laemmli 1970).

Wetblotting to Hybond™-C extra (Amersham Life Science) nitrocellulose membrane was carried out according to Towbin *et al.* (1979). After transfer, the blots were blocked in 5% w/v non-fat dried milk. Primary antibodies mouse anti-Myc monoclonal 9E10 (Roche Molecular Biochemicals) 1:15000 dilution, custom made anti-peptide antibody rabbit anti-human MTERF, (KLH-conjugated CSNDYARRSYANIKE), (Invitrogen), 1:5000 dilution, mouse Anti-FLAG® M2_antibody (Stratagene) 1:5000 dilution and mouse monoclonal antibody HA.11 (Covance, Princeton, NJ, USA) in 1:5000 dilution were used. Secondary antibodies used were peroxidase-labelled horse anti-mouse IgG (H+L, Vector Technologies Inc., affinity purified) and peroxidase-labelled goat anti-rabbit IgG (H+L, Vector Technologies Inc., affinity purified). Homemade chemiluminescence detection was carried out as described by Spelbrink *et al*. (2000) and the film (Kodak BiomaxTM ML) was exposed from 15 s to 5 min.

4.9 Immunocytochemistry

Cells cultured on coverslips were first stained for mitochondria using 100 mM MitoTracker® Red CMXRos (Molecular Probes) followed by fixation in 4% formaldehyde/5% sucrose in PBS. Cells were permeabilized for immunostaining using 0.5% Triton X-100 in PBS and blocked in 5% w/v nonfat milk. Immunodetection was carried out using primary antibodies, mouse anti-Myc monoclonal 9E10 (Roche Molecular Biochemicals) 1:1000 dilution and mouse Anti-FLAG® M2 antibody (Stratagene), 1:200 dilution. Secondary antibody was horse anti-mouse Fluorescein vector (Vector Technologies Inc, stock 1.5 mg/ml) 1:200 dilution. Coverslips were mounted on slides using Vectashield with DAPI (Vector Technologies Inc). Cells were

visualized and pictures were taken with an Olympus BX50 microscope using 100x magnification or with an Olympus IX70 inverted confocal microscope using 100x magnification.

4.10 Subcellular fractionation

Subcellular fractionation was carried out according to the protocol of Fernandez-Silva *et al.* (1996) with some modifications. Cells were harvested from 80% confluent plates. After washing with resuspension buffer (0.133 M NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 25 mM Tris-HCl pH 7.5) they were swollen in 10 mM NaCl, 1.5 mM $CaCl_2$, 10 mM Tris-HCl pH 7.5, followed by dounce-homogenization (20-25 strokes, tight-fitting pestle) on ice and breakage of the cells was verified by microscopy. An equal volume of sterile filtered sucrose/EDTA buffer (0.68 M sucrose, 2 mM EDTA, 20 mM Tris-HCl pH 7.5) was added immediately after cell lysis. Nuclei and debri were pelleted by two rounds of centrifugation at 1200 g_{max} for 10 min at 4 °C, the supernatant was re-centrifuged at 16 000 g_{max} for 30 min at 4 °C to pellet the mitochondria. The mitochondrial pellet was washed once with PBS and stored at –80 °C or lysed immediately.

4.11 Lysis of mitochondria

Mitochondria were resuspended in one volume of lysis buffer (25 mM HEPES-KOH, pH 7.6, 5 mM $MgCl_2$, 0.5 mM EDTA, 10% glycerol and freshly added 0.5 M KCl, 1 mM DTT, 0.5% Tween 20, 1 mM PMSF), lysed by pipetting up and down repeatedly until the suspension had clarified, and then by vortexing vigorously for 30 s. The lysate was incubated on ice for 5 min, and the vortexing step was repeated. The lysate was centrifuged for 45 min at 13 000 g_{max} at 4 °C. The clear supernatant was collected, carefully avoiding the fluffy layer. Protein concentration was measured using the Bradford test (Bradford 1976), using 2 µl of the supernatant.

4.12 Electrophoretic mobility shift assay (EMSA)

EMSA probes were PCR amplified using various primer pairs (See Supplementary Table 1 in I) covering several sites of interest in the human mtDNA. mtDNA was used as template for PCR and the resulting products were verified by means of sequencing. Double stranded DNA oligonucleotide probes were used for EMSA (See Supplementary Table 1 in I). Equal amounts of complementary oligonucleotide pairs were annealed to create the dsDNA oligonucleotide probes. PCR fragments and dsDNA oligonucleotide probes were labelled using T4 polynucleotide kinase (Fermentas) and [γ-32P] ATP (Amersham Pharmacia Biotech, 3000 Ci/mmol). EMSA binding reactions with minor modifications were carried out as described by Fernandez-Silva *et al.* (1996). Binding reactions contained at least 10 μl of the binding buffer (25 mM HEPES–KOH, pH 7.5, 12.5 mM $MgCl_2$, 20% glycerol, 0.1% Tween-20, 1 mM DTT), 0.2 pmol of labelled dsDNA oligonucleotide or 3 ng of labelled PCR product as probe, 5 μg of mitochondrial lysate, 100 mM KCl, 5 μg BSA and 5 μg of non-specific competitor DNA poly(dI-dC)·(dI-dC) (Amersham Pharmacia Biotech). Binding reactions were carried out at room temperature for 20 min and the tubes were placed on ice and 0.25 volume of 30% glycerol was added to terminate the binding reactions. For competition EMSA a 100-fold excess of the non-labelled competing probe was added into the reaction. Supershift EMSA reactions contained, in addition, 0.5 μg of anti-Myc monoclonal 9E10 (Roche Molecular Biochemicals), 1 μg of anti-FLAG® M2 antibody (Stratagene) or 1 μg of monoclonal antibody HA.11 (Covance, Princeton, NJ, USA). Antibody was added 30 min prior to the labelled probe. Complexes were separated and analyzed on 5–10% non-denaturing polyacrylamide TBE gels which were dried and autoradiographed using KODAK BioMax™ MS film.

4.13 RNA extraction and reverse transcriptase PCR

Total RNA for various experiments was extracted from cells using TRIzol™ Reagent (Life Technologies) according to the manufacturer's instructions and remaining traces of

DNA were removed by RNase free DNase I (Pharmacia Biotech) treatment. Ten μg of isopropanol precipitated RNA was used for cDNA synthesis using random hexamers (Pharmacia) and M-MLV reverse transcriptase (GibcoBRL), and when M-MuLV Reverse Transcriptase (Fermentas) was used five μg of isopropanol precipitated total RNA was required. Alternatively specific primers were used to synthesize specific sense and antisense transcripts of 12S, 16S, ND1 and 18S (See Supplementary Table 1 in II) using M-MuLV Reverse Transcriptase (Fermentas). cDNA for MTERF quantification was prepared using random hexamers with High Capacity cDNA Reverse Transcription Kit (AppliedBiosystems).

4.14 Quantitative RT-PCR

4.14.1 Quantitative RT-PCR using thermal cycler

Quantitative PCR was carried out using three different methods as appropriate to different experiments. cDNA was synthesized using 10 μg RNA, 0.00125 u/μl random hexamers (Pharmacia) and 200 U of M-MLV reverse transcriptase (GibcoBRL). The reaction mix was incubated for 1 h at 37 °C, boiled again for 2 min and cooled on ice for 2 min. For quantitative PCR step using MJ Research Thermo Cycler PTC-200 (GMI) 2 μl of this mixture was used in a 25 μl reaction containing the manufacturer's buffer (Finnzymes) plus 0.4 mM dNTPs (Fermentas), 0.4 μM of both primers and 2 u of Dynazyme DNA polymerase (Finnzymes). One of the primers used was specific for the expression vector (BGH: 5′ TAGAAGGCACAGTCGAGGC 3′) and the other for MTERF (MTERF465F: 5′ CGAGCAATAACACGTACTCC 3′). As quantification control, 18S was amplified in parallel using primers 18S-F (5′ TACCTGGTTGATCCTGCCAG 3′) and 18S-R (5′ TCGGGAGTGGGTAATTTGC 3′). DNaseI treated RNA was used as template for 18S PCR to study if there were any remaining traces of DNA.

4.14.2 SYBR green quantitative RT-PCR

Relative amounts of 16S rRNA, ND1 mRNA and cytosolic 18S rRNA were measured by means of SYBR Green quantitative PCR using a LightCycler™ instrument and LightCycler FastStart DNA Master SYBR Green I kit (Roche) according to the manufacturer's instructions. cDNA was synthesized using 5 μg of RNA, 40 u M-MuLV reverse transcriptase (Fermentas, protocol supplied by the manufacturer) and 0.2 μg random hexamers (Pharmacia). Three dilutions of each cDNA sample (1:10, 1:20 and 1:50) were analysed, and each reaction was performed in three technical replicates. The following primer pairs (all 5´ to 3´) and annealing temperatures were used: for 18S rRNA, 18SFOR1 – GACCATCAGATACCGTCGTA and 18SREV1 – TGAGGTTTCCCGTGTTGAGT, 52 °C; for 16S rRNA, 16Sfor1 – GGTAGAGGCGACAAACCTACCG and 16Srev1 – TTTAGGCCTACTATGGGTGT, 50 °C; for ND1, ND1for1 – GGCCAACCTCCTACTCC and ND1rev1 – GATGGTAGATGTGGCGGGTT, 50 °C. cDNA synthesized from 5 μg of RNA pooled from different cell-lines was used to prepare the standard curve, based on a five-fold dilution series. The homogeneity of all products was checked after each run by melting curve analysis.

4.14.3 Quantitative PCR using hybridization probes

To measure the relative levels of 12S, 16S, ND1, MTERF and 18S sense + antisense strand transcripts the LightCycler™ instrument was employed, together with LightCycler® FastStart DNA Master HybProbe kit (Roche). To prepare a standard curve, 5 μg of total RNA pooled from different cell lines was reverse transcribed with M-MuLV Reverse Transcriptase (Fermentas) using specific primers (TIB MOLBIOL) (see Supplementary Table 1 in II for list of primers and probes used). Three dilutions (1:10, 1:20 and 1:50) were analysed from each cDNA. After 10 min of initial denaturation at 95 °C, the cycling conditions (45 cycles) were the following: denaturation at 95 °C for 10 s, annealing at T_M-5 °C depending on the primer for 10 s, and elongation at 72 °C for (length of the amplified fragment bp/25 bp) s. Samples were also electrophoresed in

agarose gel to ensure that the single amplified product was of the correct size. In addition, melting curve analysis was used to ensure the purity of the amplified PCR product. For normalization of expression levels, the expression of 18S rRNA was measured. The relative expression of each gene was calculated by dividing the value by the 18S value of each sample.

4.15 EtBr-induced depletion of mitochondrial RNA + DNA

To deplete mtDNA from cells EtBr was added in 60 x 15 mm plates at ~50% confluence. 250 ng/ml of EtBr was added and the cells were cultured for a further 48 h then passaged at different densities so that plates were always ~70-80% confluent when harvested. Samples were collected before adding EtBr (day -2), on the day when drug was washed away (day 0) and 24, 48, 72, 96 and 120 h after removing EtBr. EtBr was washed away by changing the medium 3 and 6 h after passaging the cells on day 0 and then every day. RNA was extracted using TRIzol™ Reagent (Life Technologies). When the effect of silencing of MTERF was being studied on the transcript levels during the recovery period following EtBr treatment, the cells were transfected with siRNA on day -2 before adding EtBr and transfected again on day 2.

Alternatively, when suppressing mtDNA copynumber and mitochondrial transcription in TFAM over-expressing cells, the cells were treated with 50 ng/ml EtBr for 72 h and then washed and replated in fresh medium followed by further 48 h in culture.

4.16 Northern blot quantification of RNA

4.16.1 Northern blotting using neutral acrylamide/urea gel electrophoresis

Total RNA samples were electrophoresed at 4 °C overnight at 100 V in neutral 12% acrylamide/7 M urea gels in TBE buffer and electroblotted onto Zeta-Probe GT membrane (BIO-RAD) at 4 °C first at 15 V for 30 min and then at 30 V for 60 min in 1xTBE buffer. RNA was u.v.-crosslinked to the membrane using Kodak UltraLinker.

Oligonucleotide probes were labelled using T4 polynucleotide kinase (PNK, MBI Fermentas, protocol supplied by the manufacturer) and [γ-32P] ATP (Amersham Pharmacia Biotech, 3000 Ci/mmol). The probes were as follows: 5S (5´-GGGTGGTATGGCCGTAGAC -3´), $tRNA^{Leu(UUR)}$ (5´- GTTTTATGCGATTACCGGGC -3´) and $tRNA^{Phe}$ (5´- CTAAACATTTTCAGTGTATTGC -3´). The effect of modified TFAM level on mitochondrial transcripts was studied also using ND3 (5´-GTCACTCATAGGCCAGACTT -3´) and $tRNA^{Tyr}$ (5´-ATTTACAGTCCAATGCTTCACTC -3´) probes as previously described by Toompuu *et al*. (2002). Labelled probes were purified using mini Quick Spin Columns (Roche) according to the manufacturer's protocol.

Blots were prehybridised in buffer containing 50 mM PIPES, 100 mM NaCl, 50 mM $NaH_2PO_4 \cdot H_2O$, 1 mM EDTA, 5% SDS pH 6.8 and 0.1 mg/ml of herring sperm DNA (hsDNA) for two hours at 42 °C. Hybridization was continued overnight after adding the labelled probe into the prehybridization solution. After hybridization the membranes were washed at 42 °C with 2 x SSC - 0.1% SDS (20 x SSC = 3 M NaCl, 0.3 M sodium citrate) twice for 5 min and then once for 10 min with 1 x SSC - 0.1% SDS. Membranes were exposed to X-ray film (Kodak BioMax MS film) at -80°C and autoradiographic signal was quantified by phosphorimaging (Phosphorimager SI, Molecular Dynamics). Probe was washed away from the membranes by stripping them in boiling 0.5% SDS solution for 3 min.

4.16.2 Northern blotting using formaldehyde/agarose gel electrophoresis

Formaldehyde agarose gel electrophoresis and subsequent hybridization were carried out as described previously by El Meziane *et al*. (1998). 16S rRNA or ND1 probes used for Northern blotting were labelled by random-priming as described earlier by Toompuu *et al*. (1999). The ND1 and 16S probes were synthesized according to Toompuu *et al*. (1999) by *Apa*I digesting the amplified fragment to produce the two probes.

4.17 Systematic evolution of ligands by exponential enrichment (SELEX)

Randomized DNA ligand library was created according to Blackwell (1995) with minor modifications. The 46 nt long oligonucleotide template contained 14 randomized nucleotides in the middle and fixed ends of 16 nt matching with the primers coding recognition site either for *Bam*HI or *Eco*RI. Klenow fragment (Fermentas) was used to synthesize the second strand and dsDNA ligand library was gel-purified from an EtBr-stained 14% native polyacrylamide gel using the QIAEX kit (QIAGEN, protocol supplied by the manufacturer). Ligand selection was carried out as described for EMSA binding reactions with the exception that 10 μg of mitochondrial protein lysate from epitope-tagged protein of interest expressing Flp-In™ T-REx™-293 cells, 0.8 μg of the ligand DNA and 6.25 μg of non-specific competitor DNA poly(dI-dC)·(dI-dC) were used. Pre-swollen antibody linked Sepharose beads (Amersham Biosciences) were washed twice with EMSA binding buffer lacking poly(dI-dC)·(dI-dC) and resuspended in 1.5 volumes of the same buffer containing now also poly(dI-dC)·(dI-dC). Antibody-linked beads were added to binding reactions and after this selection step, beads were washed in poly(dI-dC)·(dI-dC) containing EMSA buffer, followed by seven washes in the buffer lacking poly(dI-dC)·(dI-dC). Proteins were digested by proteinase K (Fermentas) overnight and DNA was recovered by phenol extraction. After ethanol precipitation DNA ligands were resuspended in H_2O and used as template for PCR using SELEX primers (5´ - GGTGAATTCGCTCACG - 3´and 5´- GAACGGATCCCTTTCG – 3´, restriction sites

for cloning underlined) and Pfu DNA polymerase (Promega). The enriched ligand DNA was gel-purified as above. After seven cycles of selection and enrichment, the enriched ligand DNA was cloned into pCR®4Blunt-TOPO® vector (Invitrogen). Clones were sequence verified using standard primers on an ABI 3100 sequencer using the BigDye® Terminator v3.1 cycle sequencing kit (AppliedBiosystems).

4.18 Chromatin immunoprecipitation

Mitochondrial DNA immunoprecipitation (mIP) was carried out according to Lu *et al*. (2007) with some modifications. Protease inhibitor used in lysis buffer was Complete, Mini protease inhibitor cocktail (Roche). The mtDNA was sheared to fragments using Sonics Vibra-Cell sonicator, 3 mm tip at 25% power for 3 × 20 s (1 s on, 1 s off) and incubated on ice for 30 s between to produce DNA fragments of approximately 500–600 bp. Pre-swollen Protein A Sepharose (Amersham Biosciences) beads were added to lysates to pre-clear them. For immunoprecipitation 5 µg of mouse anti-Myc monoclonal 9E10 (Roche Molecular Biochemicals) or anti-FLAG® M2 antibody (Sigma) was used per reaction. Immunoprecipitation was carried out overnight at 4°C. See Supplementary Table I in paper I for the primers used for the mIP PCR.

4.19 mtDNA copynumber assay

Total DNA was extracted from cultured human cells as described by Sambrook *et al*. (1989) for mtDNA copynumber analysis. Copynumber was determined by means of quantitative PCR using ABI Prism 7000 (AppliedBiosystems) with amyloid precursor protein (APP) as a single-copy nuclear DNA standard. Following primers and probes were used (all shown in 5´-3´ orientation): APP Forward: TTTTTGTGTGCTCTCCCAGGTCT, APP Reverse: TGGTCACTGGTTGGTTGGC, APP Probe (FAM+BHQ): CCCTGAACTGCAGATCACCAATGTGGTAG, Cyt-b Forward: GCCTGCCTGATCCTCCAAAT, Cyt-b Reverse: AAGGTAGCGGATGATTCAGCC, Cyt-b Probe (TET+BHQ):

CACCAGACGCCTCAACCGCCTT. Analysis of the data was carried out using the software supplied by the manufacturer (AppliedBiosystems).

4.20 Isolation of mtDNA

For mtDNA isolation mitochondria were isolated according to Spelbrink *et al*. (2000). mtDNA (mitochondrial nucleic acids) was extracted for 2DNAGE from cultured cells as described in IV.

4.21 Two dimensional neutral agarose gel electrophoresis

4.21.1 Enzymatic treatments of mtDNA

For 2DNAGE analysis one µg aliquots of total mitochondrial nucleic acids were digested with a restriction enzyme to produce the fragment of interest. *NheI*, *Bcl*I, *Hinc*II and *Pvu*II (Fermentas) and *Acc*I and *Bam*HI (New England Biolabs (Finnzymes)) were used for digesting mtDNA. Restriction enzymes were used according to protocols supplied by the manufacturers with one exception; digestion with *Bcl*I was allowed to continue for double the recommended time. In certain experiments the restriction enzyme digestion was followed by ethanol precipitation and digestion with 50 U of S1 Nuclease (Promega). S1 nuclease digestion was terminated by phenol extraction.

4.21.2 Two dimensional neutral agarose gel electrophoresis and Southern blotting

2DNAGE was generally carried out as described by Friedman and Brewer (1995). To study restriction intermediates of different sizes, different gel conditions were applied for 2DNAGE (Table 4.1). Electrophoresis in the first dimension (1D) was carried out in TBE buffer without EtBr and the gel was stained with EtBr (300 ng/ml) only after

electrophoresis. The first dimension separates DNA molecules in proportion only to their mass. The mtDNA lanes were cut out exposing the mtDNA to u.v.-light as briefly as possible. The cut lanes were positioned at a 90° angle in the gel tray and the second dimension (2D) agarose gel, cooled to 55°C, was cast around them. The second dimension agarose gel contains 300 ng/ml EtBr and the migration of replication intermediates is thus strongly affected by their shape. Electrophoresis of shorter fragments was carried out at 4 °C and the electrophoresis of full genome-length mtDNA fragments, when a restriction enzyme cutting mtDNA only once had been used, was performed at room temperature. After second dimension electrophoresis Southern blotting was carried out, transferring DNA by capillary action to Hybond N nylon membrane (Amersham) overnight. Before blotting, the gels were soaked in 0.25 M HCl for 20 min, rinsed with water, denatured in 0.5 NaOH, 1.5 M NaCl for 2 x 20 min and neutralized in 1.0 M Tris-HCl pH 7.2, 2.0 M NaCl.

Table 4.1 Different gel conditions for 2DNAGE.

Fragment length	Dimension	Gel (agarose %)	Voltage (V/cm)	Temp (°C)	Time (h)
< 2 kb	First	0.6	1.2	4	20
	Second	1.4	9	4	6
2 – 5 kb	First	0.4	1.8	4	20
	Second	1.0	10	4	6
16.6 kb	First	0.28	1.7	Room Temp	24
	Second	0.58	1.8	Room Temp	67

4.21.3 Interpretation of 2D gels

2DNAGE gels were generally interpreted according to Friedman and Brewer (1995). For a typical 2D gel mtDNA is first digested with a restriction enzyme and DNA fragments are separated on the basis of their mass in the first dimension electrophoresis and on the

basis of their shape in the second dimension electrophoresis as described above. When transferred to membrane the fragment of interest is detected with a suitable probe. Figure 4.1 shows how non-replicating molecules form a shallow arc of linears (linear arc). The 1n spot consists of nonreplicating molecules on the arc of linears that are the size of the fragment. Replicating molecules migrate above the shallow arc of linears and form the simple Y, bubble or double-Y arcs. Also an X-spike (X-arc) can be seen which is formed by X-shaped replication or recombination intermediates. On the simple Y arc a replication fork stall can be seen as a replication pause site visualized as a discrete spot.

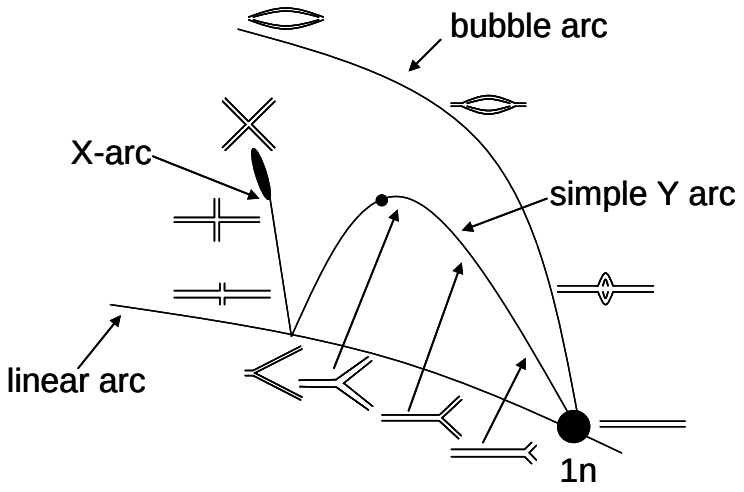

Figure 4.1. Interpretation of a 2 dimensional neutral agarose gel.

5. Results

5.1 Functional studies on mitochondrial transcription termination factor MTERF (I, II)

5.1.1 Over-expressed MTERF is mitochondrially targeted in cultured human cells (I)

HEK293-derived cells were established to express both epitope-tagged MTERF as well as natural MTERF in order to study the functional role of MTERF in mitochondrial DNA maintenance. MTERF was observed to be efficiently over-expressed in cultured human cells (Figure 5.1). As MTERF has been reported to be a mitochondrial protein the mitochondrial targeting of the epitope-tagged MTERF was checked by means of immunocytochemistry. HEK293T cells were either stably or transiently transfected with MTERF-MycHis. A filamentous staining pattern typical for mitochondria with no nuclear staining was seen by means of immunocytochemistry therefore demonstrating MTERF to be mitochondrially localized (Figure 5.2).

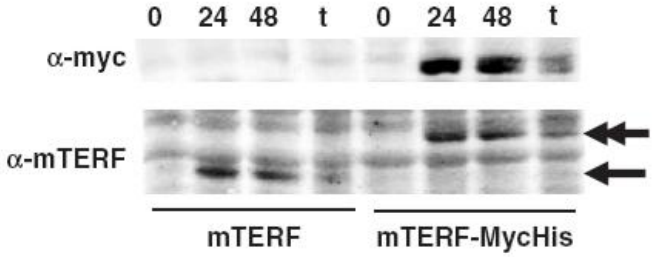

Figure 5.1. Over-expression of MTERF and MTERF-MycHis in cultured human cells. MTERF or MTERF-MycHis transfected Flp-In™ T-REx™-293 cells were induced for expression as described (0, 24, 48 h) and HEK293T cells were transiently transfected (t). Western blots were probed with anti-Myc or anti-MTERF antibodies. The endogenous MTERF protein detected by the anti-MTERF antibody is marked with one arrow and the MTERF-MycHis fusion protein detected by anti-Myc antibody is indicated with double arrows. Reprinted from original article (I), copyright (2007), by permission of Oxford University Press.

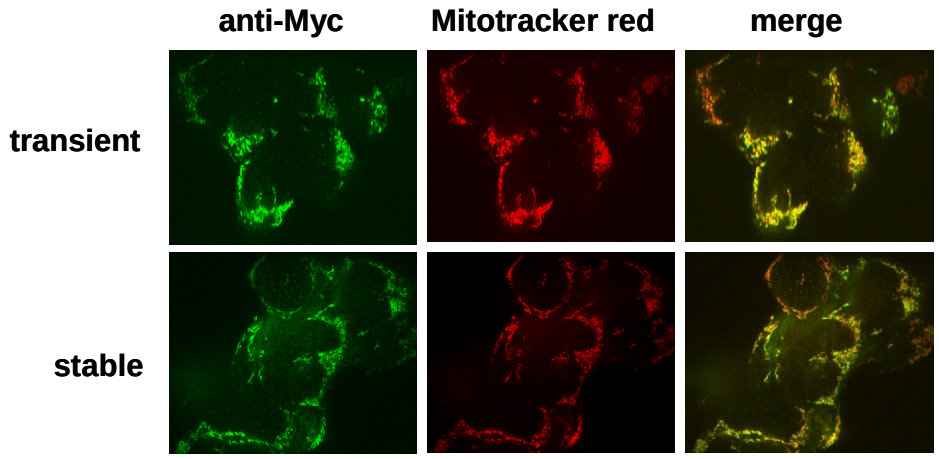

Figure 5.2. Subcellular localization of MTERF-MycHis in HEK293T cells. HEK293T cells were either transiently or stably transfected with MTERF-MycHis and detection was carried out by anti-Myc monoclonal antibody and counterstained with Mitotracker Red. Reprinted from original article (I), copyright (2007), by permission of Oxford University Press.

5.1.2 MTERF efficiently binds mtDNA (I, II)

Previously MTERF has been shown to be a DNA-binding protein having binding affinity to a 28 bp sequence in the $tRNA^{Leu(UUR)}$ gene which is in downstream and adjacent to the 16S rRNA gene (Kruse *et al.* 1989). Therefore we tested whether over-expressed MTERF as well as epitope-tagged MTERF are also able to bind DNA (Figure 5.3). Studying the effect of over-expressed MTERF on its binding to DNA by means of EMSA, using probes covering the reported canonical MTERF binding site together with mitochondrial protein lysate is also relevant to later studies of the effects of modulating MTERF levels on human mtDNA replication *in vivo*. Because crude mitochondrial protein extracts were used, other proteins present may have an effect on MTERF binding in these experiments. Over-expression of MTERF in any manner tested, transient and stable transfection or under doxycyclin induction, resulted in a clear increase in the binding of MTERF to its target site (Figures 5.3, 2C in I, Supplementary Figure S1B in II).

Cell clones stably transfected with MTERF were tested for MTERF over-expression by means of EMSA, and the cell clones showing the highest expression were chosen for further study (Supplementary Figure S1B in II).

The binding of MTERF to its canonical binding site was shown to be specific by EMSA supershift. The Myc epitope-tagged MTERF protein was supershifted by anti-Myc antibody but not with anti-FLAG antibody. Also the signal was competed out when excess unlabelled probe was added to the reaction mix (Figure 5.3).

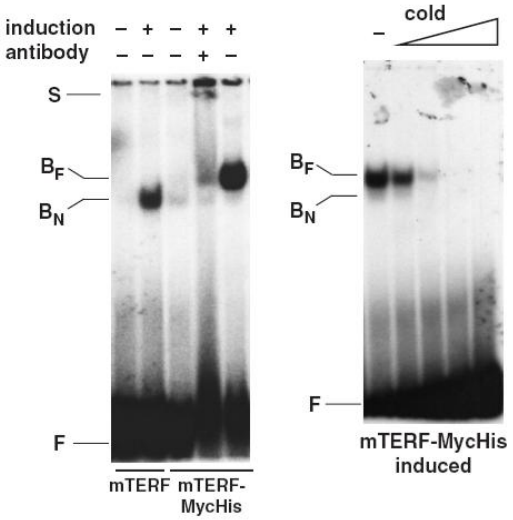

Figure 5.3. MTERF binding to human mtDNA. MTERF or MTERF-MycHis transfected Flp-In™ T-REx™-293 cells were induced for protein expression and EMSA was carried out using Leu-short dsDNA oligonucleotide probe and mitochondrial protein lysates from the cells. Supershift EMSA was carried out using anti-Myc antibody and competition EMSA using 1-, 10-, 100- and 1000-fold mass excess of Leu-short dsDNA oligonucleotide competitor ('cold') or without competition. F denotes free probe, B_N denotes for natural MTERF complexes and B_F the MTERF-MycHis fusion protein complexes. S denotes anti-Myc antibody supershifted complex. Reprinted from original article (I), copyright (2007), by permission of Oxford University Press.

5.1.3 Silencing MTERF by means of RNAi (I)

To study the effect of knocking down MTERF, using the RNA interference (RNAi) method, I tested several siRNAs targeting MTERF for silencing efficiency, using stably transfected MTERF-MycHis over-expressing cells in order to be able to determine the knockdown efficiency at protein level using Western blotting. siRNA molecule MTERF.1 was observed to lead to efficient silencing of MTERF when studied by means of Western blotting (Figure 5.4A) or immunocytochemistry (Figure 5B in I). When the effect of silencing MTERF on DNA-binding activity was studied by means of EMSA 48

h after the transfection of cells with the siRNAs, it was estimated that knock-down lead to loss of ~90% of the protein based on densitometry of the autograph signals (Figure 5.4B).

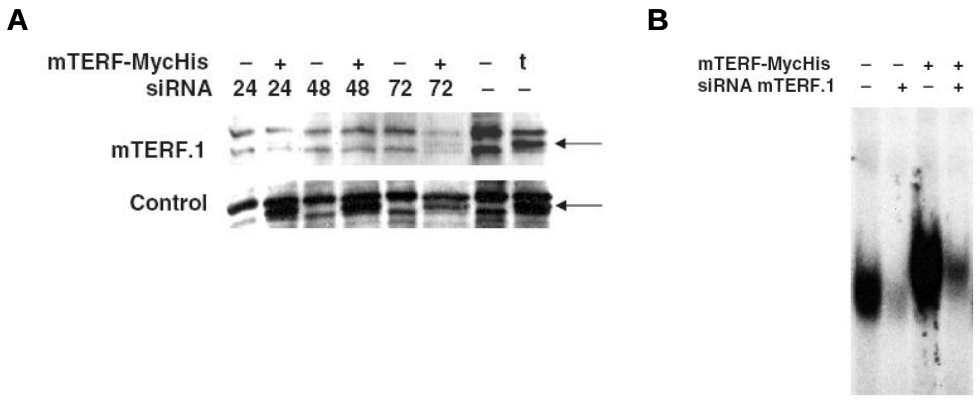

Figure 5.4. Silencing MTERF by means of RNAi. A) MTERF was knocked down using siRNA mTERF.1 and the silencing efficiency versus siRNA control (Control) was checked by means of Western blotting. Cells that were used were either untransfected (-), transiently transfected (t) or stably transfected with MTERF-MycHis expression construct. Cells were harvested 24, 48 and 72 h after transfection with the siRNA. MTERF-MycHis fusion protein is denoted with arrows. MTERF-MycHis fusion protein migrates between two background bands which are present in all western blots and thus these bands serve as internal loading control. The penultimate lane in the upper panel showing the untransfected, non-siRNA-treated cells is approximately 3-fold overloaded. B) EMSA was carried out using mitochondrial protein extract from HEK293T cells +/- stable transfection with MTERF-MycHis. Cells were harvested 48 h after being transiently transfected with or without MTERF.1 siRNA. Leu-short dsDNA oligonucleotide probe was used in EMSA. The experimental conditions in this figure are the same as in Figure 5.3, but here the amount of the background signal and the exposure time are different. Reprinted from original article (I), copyright (2007), by permission of Oxford University Press.

5.1.4 Effects of over-expressing MTERF on mtRNA synthesis (II)

5.1.4.1 Steady state RNA levels are not affected by MTERF over-expression

The relative activities of the two transcription units of the mtDNA heavy strand, namely PH1 and PH2 transcription units, were studied in MTERF stably over-expressing HEK293T cell clones during normal growth. The relative activities of these two transcription units can be determined by means of Northern blot analysis measuring the steady-state level of transcripts within different transcription units, here $tRNA^{Leu(UUR)}$ and ND1 mRNA for the PH2 transcription unit, and $tRNA^{Phe}$ plus 16S rRNA for the PH1 transcription unit.

I studied the $tRNA^{Phe}$/$tRNA^{Leu(UUR)}$ ratio in MTERF over-expressing cell clones compared to cells transfected with empty expression vector, normalized using 5S rRNA as a loading control. I established that there is no significant difference in the ratio of $tRNA^{Phe}$/$tRNA^{Leu(UUR)}$ in different cell clones over-expressing the natural variant of MTERF compared to the control cells, cells transiently transfected with the MTERF over-expression construct or mock transfected cells (Figure 5.5A). The ratio of $tRNA^{Phe}$ to cytosolic 5S rRNA, which represents the global amount of mitochondrial transcription, was more variable, but there was no systematic relationship with MTERF over-expression (Figure 5.5A).

I used Q-RT-PCR to determine the mature ND1 mRNA and 16S rRNA levels in inducible Flp-In™ T-Rex™-293 cells stably transfected with the MTERF-MycHis construct compared to non-induced cells. There was no significant difference in the relative amounts of mature 16S rRNA and ND1 mRNA observed (Figure 5.5B). There was no difference between MTERF over-expressing clones and control cells on the basis of Northern blots: see Figure 5.6C and compare lanes 1 of panels *i* and *ii* (control cells) with lanes 1 of panels *iii* and *iv* (over-expressor cells).

5.1.4.2 Over-expression of MTERF does not affect mitochondrial tRNA levels during the recovery period after EtBr depletion

MTERF stably over-expressing and control cells were depleted of mitochondrial RNA and DNA with EtBr for 48 h. Our reasoning was that the possible effect of MTERF protein level modification on mitochondrial tRNA synthesis would be clearer during the recovery period when new mtDNA is synthesized and transcribed in large amounts. Cells were allowed to recover over 5 days during which tRNA levels were measured at regular intervals. The $tRNA^{Phe}$/ $tRNA^{Leu(UUR)}$ ratio was determined by means of Northern blot analysis. MTERF over-expression was not observed to alter the mitochondrial RNA levels during recovery from mtDNA depletion (Figure 5.5C).

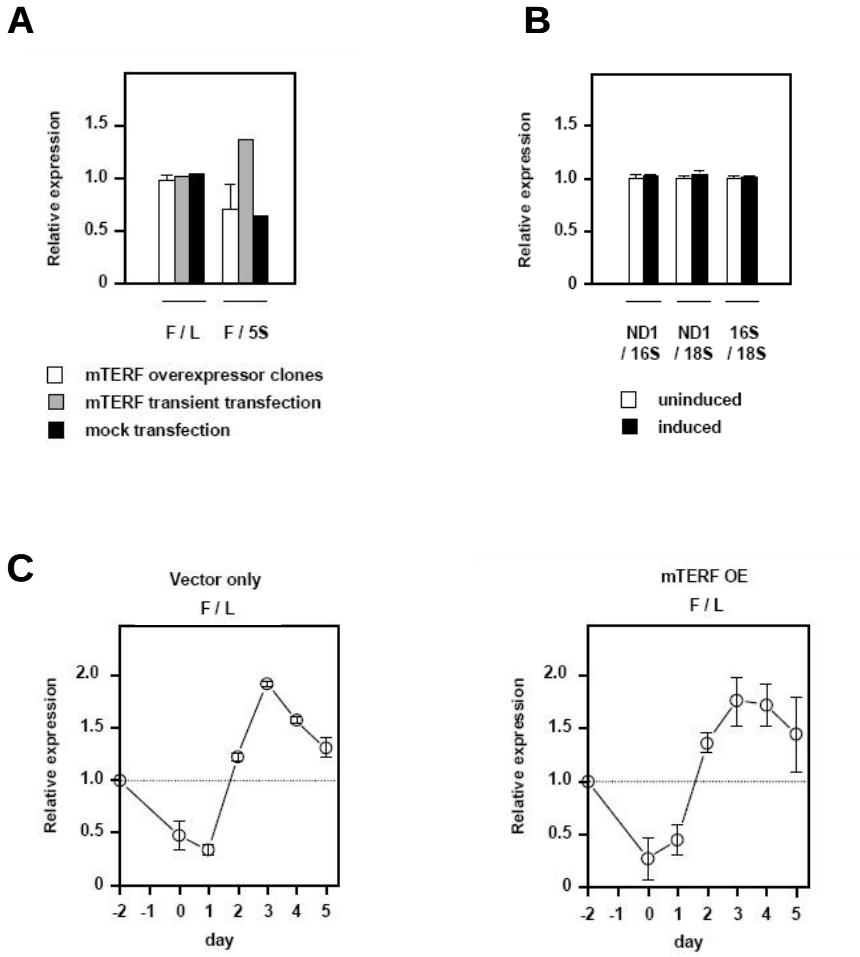

Figure 5.5. The effect of over-expressing MTERF on mitochondrial RNA levels. A) Northern blot signal ratios of $tRNA^{Phe}$ to $tRNA^{Leu(UUR)}$ (F/L) and $tRNA^{Phe}$ to 5S rRNA (F/5S) for MTERF-over-expressing clones, normalized to the corresponding ratio in cells stably transfected with empty-vector. Shown here alongside are the HEK293T cells transiently transfected with the same MTERF construct or mock-transfected, both single reference experiments. B) Mitochondrial transcript and cytosolic 18S rRNA levels measured by means of Q-RT-PCR 3 days after induction in Flp-In™ T-Rex™-293 cells stably transfected with MTERF-MycHis versus non-induced cells. Data normalized, in each case, to the relevant ratio for non-induced cells. C) Northern blot analysis of cells stably transfected with empty vector or an MTERF over-expressor clone (OE), probed with mitochondrial $tRNA^{Phe}$ and $tRNA^{Leu(UUR)}$ and normalized to the ratio at timepoint -2 d. EtBr was added on day -2, washed away on day 0, and the cells allowed to recover over 5 days. Reprinted from original article (II), copyright (2010), by permission of BioMed Central.

5.1.5 Effects of silencing MTERF on mtRNA synthesis (II)

5.1.5.1 Effects of MTERF knockdown on steady-state mature mitochondrial RNA levels

Over-expression of MTERF is not necessarily enough to cause any effects on mitochondrial RNA synthesis because according to Micol *et al.* (1997) the MTERF target binding sites are up to 80% occupied *in vivo*. Therefore an opposite approach was used and MTERF expression was down-regulated by RNA interference (RNAi) using short interfering RNAs (siRNA) (Elbashir *et al*. 2001a, Elbashir *et al*. 2002b) in HEK293T cells and also in MTERF-MycHis over-expressing cells.

The effect of MTERF knockdown on the $tRNA^{Phe}/tRNA^{Leu(UUR)}$ ratio was studied 7 days after the first siRNA transfection by means of Northern blot. siRNA transfection was repeated on day 4. When MTERF-MycHis over-expressing cells were studied, there was no significant difference between the siRNA-treated and the control cells. HEK293T cells showed a slight decrease on the $tRNA^{Phe}/tRNA^{Leu(UUR)}$ ratio due to MTERF knockdown, with a modest increase on the levels of both mitochondrial tRNAs compared with 5S rRNA relative to untreated cells (Figure 5.6A). Mature 16S rRNA and ND1 mRNA levels were determined as well and they were found to be unaffected in both cases (Figure 5.6C, compare lanes 1 and 2 of panels *i* and *ii* and lanes 1 and 2 of panels *iii* and *iv*). As tRNA and rRNA steady-state levels are rather stable they do not necessarily correlate well with the rates of their transcription. mRNA transcripts are generally less stable i.e. ND1 mRNA levels might correlate better with its transcription rate.

5.1.5.2 Knocking down MTERF does not affect relative mitochondrial tRNA levels during the recovery period after EtBr induced depletion

We next studied whether the re-accumulation of mitochondrial transcripts is affected by MTERF knockdown after depletion of mitochondrial RNA and DNA with EtBr. Cells were silenced for MTERF prior to treatment with EtBr on day -2 and then again on day 2.

EtBr treatment was carried out from day 0 for 48 h, after which it was washed away and cells were subsequently washed daily. MTERF silenced cells did not differ from mock-transfected cells when the relative mitochondrial RNA levels were studied after the EtBr-induced depletion of mitochondrial RNA species (Figure 5.6B).

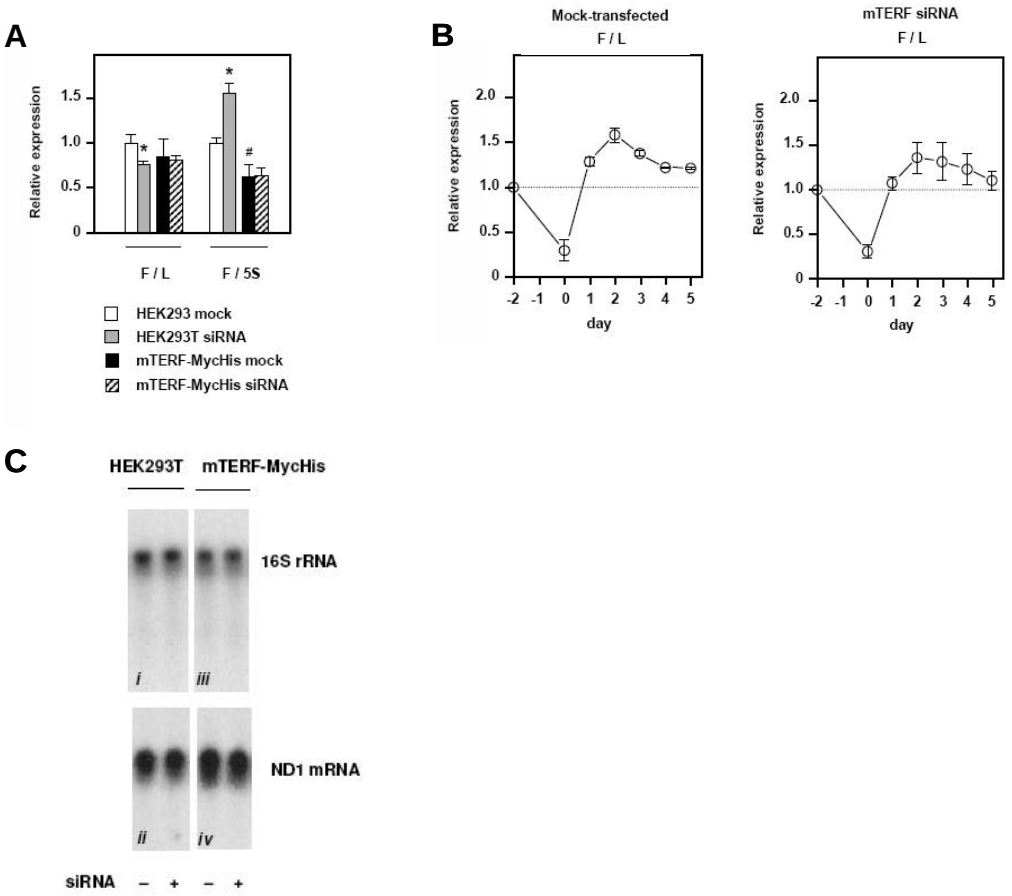

Figure 5.6. The effect of silencing MTERF on mitochondrial RNA levels. A) MTERF was knocked down and the relative activities of the transcription units were calculated from Northern blot data and normalized to the corresponding ratio of the mock-transfected cells. Statistically significant difference (*) from the corresponding mock-transfected cells (*t*-test, $p<0.05$) and significant difference (#) between cell lines (*t*-test, $p<0.01$). Original blots shown in Figure S1C in II. B) Northern blot signal ratios for RNA of MTERF knock down and mock-transfected cells, probed for mitochondrial $tRNA^{Phe}$ and $tRNA^{Leu(UUR)}$ and normalised to the ratio at timepoint -2 d. C) Northern blot, probed for 16S rRNA and ND1, of RNA from cells knocked down for MTERF or mock transfected (-). Adjacent panels are from the same exposure of the same gel. Reprinted from original article (II), copyright (2010), by permission of BioMed Central.

5.1.6 Altering MTERF levels affects both sense- and antisense-strand transcription (II)

In order to analyze total sense and antisense transcripts from either side of the MTERF binding site, cDNA was prepared using specific primers for sense and antisense strand synthesis. The effect of manipulating MTERF levels was studied in three different cases; first, cells stably transfected with MTERF were compared to control cells transfected with the empty vector; second, MTERF was over-expressed in inducible Flp-In™ T-Rex™-293 cells and this was compared to non-induced cells; and third, MTERF was knocked down using shRNA. The positions of the primers used for Q-RT-PCR are shown in Figure 5.7A. When MTERF was knocked down it was observed that the amount of anti-16S RNA increased dramatically whereas when MTERF over-expression was induced in Flp-In™ T-Rex™-293 cells the anti-16S:anti-ND1 ratio was decreased in a statistically significant manner. The same effect was seen in cell clones stably transfected with MTERF: the anti-16S:anti-ND1 ratio was decreased but the effect was statistically significant only for one of the two clones studied (Figure 5.7B).

Induced over-expression of MTERF yielded 20-fold increase in MTERF mRNA level (Figure 5.7E) and MTERF knockdown produced a decrease by a factor of 2 at the RNA level (Figure 5.7F). Induced over-expression caused a dramatic decrease in 16S antisense transcripts and also a small decrease in the ND1 antisense transcripts (Figure 5.7C). MTERF knockdown produced mainly a significant decrease in the ND1 antisense transcripts (Figure 5.7D). Clearly, over-expression and knockdown of MTERF led to different changes in each RNA transcript when comparing treated cells to untreated control cells.

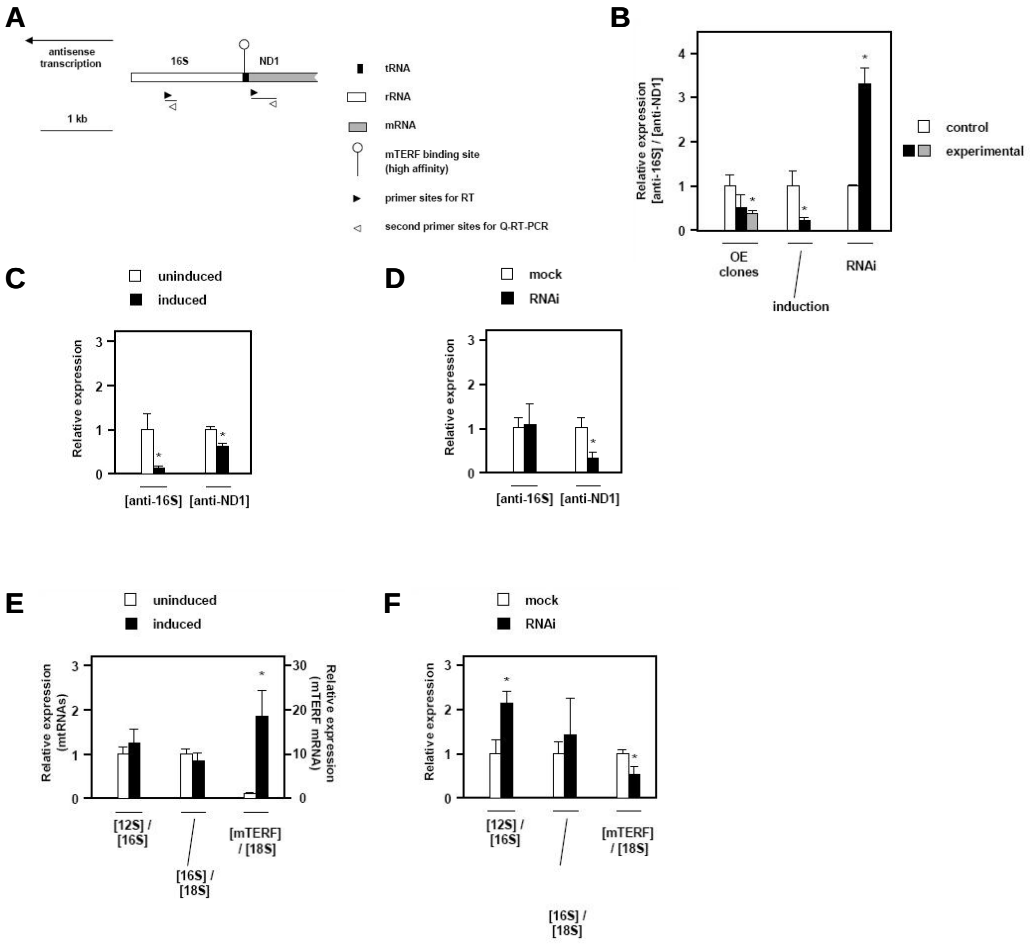

Figure 5.7. Modulating MTERF levels affects mitochondrial antisense RNA levels. A) Positions of the primers designed for Q-RT-PCR. B) Q-RT-PCR analysis: two MTERF over-expressing clones (OE) versus cells transfected with empty vector: MTERF expression construct-transfected doxycyclin-induced Flp-In™ T-Rex™-293 cells versus non-induced cells; and MTERF-targeted shRNA transfected cells versus mock-transfected cells, tested for relative steady-state levels of anti-16S and anti-ND1 transcripts. Data normalized to corresponding control cells. Statistically significant difference (*) compared to control cells (t test, $p<0.02$). C) Data from panel B was replotted to study relative changes in anti-16S and anti-ND1 transcript levels separately for Flp-In™ T-REx-293 cells induced to express mTERF. D) Data from panel B was replotted to study relative changes in anti-16S and anti-ND1 transcript levels separately for HEK293T cells transfected with mTERF-targeted shRNA *versus* mock-transfected cells. 18S was used as internal normalization standard and data were normalized to values of relevant untreated control cells. E) Q-RT-PCR with hybridization probes (probe sets T1, R2, C1 shown in Table S1 in II) to study relative steady-state levels of 12S, 16S and 18S sense-strand transcripts and of mTERF mRNA over 18S rRNA (probe sets M1 and C1 shown in Table S1 in II) for Flp-In™ T-REx-293 cells induced to express mTERF. F) Q-RT-PCR with hybridization probes (probe sets T1, R2, C1 shown in Table S1 in II) to study relative steady-state levels of 12S, 16S and 18S sense-strand transcripts and of mTERF mRNA over 18S rRNA (probe sets M1 and C1 shown in Table S1 in II) for HEK293T cells transfected with mTERF-targeted shRNA *versus* mock-transfected cells. * indicates statistically significant differences between treated and non treated cells (*t* test, $p < 0.02$). Reprinted from original article (II), copyright (2010), by permission of BioMed Central.

Different control experiments were carried out to validate the findings. Another primer/probe set (Figure S3B in II) was used to confirm the main findings and was also used to study the effects on the relative 16S rRNA and ND1 sense transcript levels, which were observed not to vary due to these manipulations (Figure S3C in II). This was supported by the findings from the analysis of mature transcripts by Northern blots (Figure 5.5B, 5.6C). Also sense transcript level of 12S rRNA (in PH1 transcription unit) relative to cytosolic 18S rRNA was studied in treated versus untreated cells (Figure 5.7E and F). The effects were also analyzed on the mitochondrial 16S rRNA sense transcript level relative to cytosolic 18S rRNA (Figure 5.7E and F). When MTERF was knocked down there was a significant increase in sense-strand 12S rRNA steady-state transcripts (Figure 5.7F) whereas MTERF over-expression did not have any effects on the sense-strand transcripts of 12S rRNA and of 16S rRNA relative to each other or to cytosolic 18S rRNA (Figure 5.7E).

5.1.7 MTERF has multiple novel binding sites in mtDNA (I)

I studied the binding of MTERF to mtDNA by means of EMSA using protein lysates from crude mitochondrial extracts from MTERF over-expressing cells. Having already shown that over-expression of MTERF leads to a large increase of binding at the canonical biding site, it was established that both natural MTERF and the MycHis epitope-tagged variant were able to bind mtDNA efficiently with the chosen EMSA conditions. I then designed 150 bp long EMSA probes overlapping each other to cover the entire non-coding region of the genome (NCR) and adjacent fragments, as well as the region at OL, the IQM tRNA cluster, the ND1 gene between the IQM tRNA cluster and the canonical MTERF binding site within the $tRNA^{Leu(UUR)}$ coding gene, the ATPase6 coding sequence and the ATPase6/COIII gene junction site, plus other regions of the genome. A full list of the probes used is presented in Supplementary Table 1 in I.

Binding was scored as positive if EMSA signals were enhanced by both natural MTERF and MTERF-MycHis, if the complexes migrated at slightly different positions

due to the presence of the MycHis epitope tag and if the MTERF-MycHis complexes were shifted by the anti-Myc antibody. The strength of the binding was determined by actual competition experiments or it was simply based on estimating the EMSA signal by eye. I identified a cluster of four novel binding sites for MTERF, which I classified as moderately strong, within the ND1 coding gene and the adjacent tRNA cluster, located within the probes ND1.1, ND1.2 and IQM2 in addition to the previous 'canonical' binding site in the $tRNA^{Leu(UUR)}$ (Figure 5.8A and 5.8C). When MTERF binding to the latter was competed with unlabelled ND1.1 or vice versa, it was estimated that MTERF binds ND1.1 with an affinity that is 1-2 orders of magnitude weaker than that at the $tRNA^{Leu(UUR)}$ gene (Figure 5.8B). Shorter overlapping fragments were created to cover the ND1.1 fragment in order to study the binding within this fragment in more detail (Supplementary Figure 2B in I). This analysis revealed evidence for two distinct binding sites within the ND1.1 fragment (Supplementary Figure 2D in I).

I identified four other binding sites within the D-loop part of the NCR, one at O_L, one in OH1 fragment adjacent to O_H (Figure 5.8A, 5.8C, Supplementary Figure 2E in I). In addition I found one possible binding site within the OH5 fragment (Figure 5.8C, Supplementary Figure 2E in I).

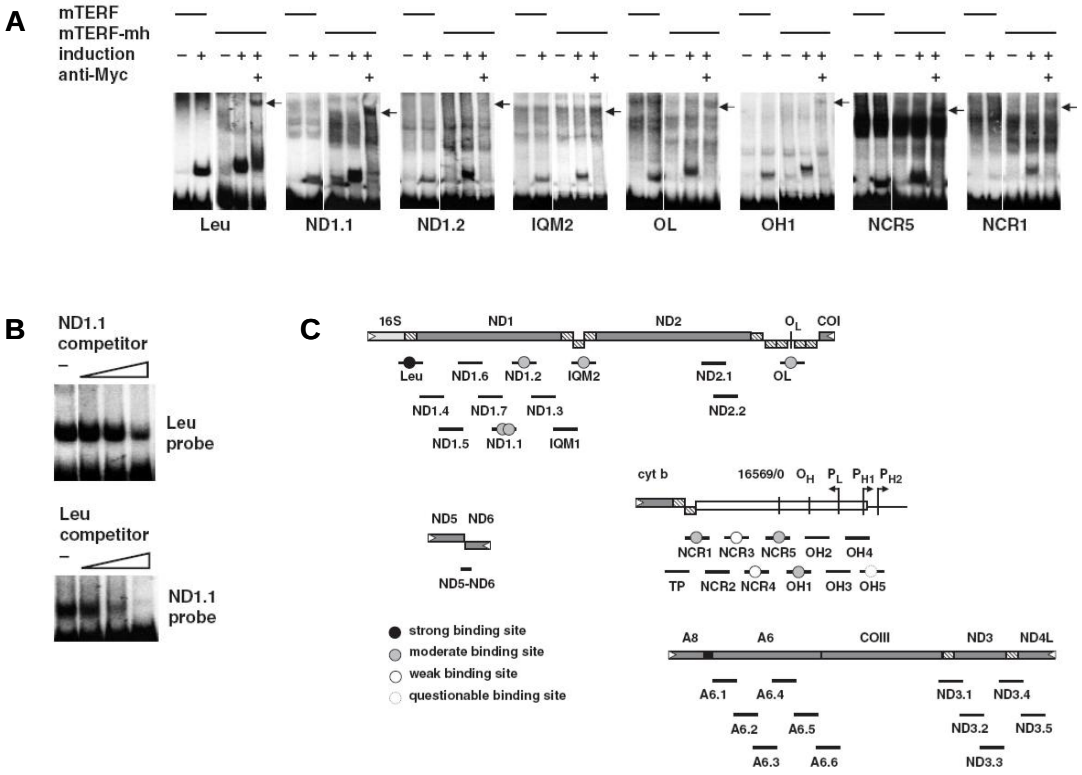

Figure 5.8. MTERF binding to human mitochondrial DNA sequences in vitro. A) EMSA using 150 bp long probes and mitochondrial protein extracts from either induced or non-induced natural MTERF or MTERF-MycHis transfected Flp-In™ T-Rex™-293 cells. Supershift was carried out with anti-Myc monoclonal antibody. Anti-FLAG antibody did not yield supershift nor inhibit the formation of complexes when used as a control. B) Competition EMSA with mitochondrial protein extract from MTERF-MycHis over-expressing cells using either 1x, 10x or 100x mass excess of the cold competitor over the hot probe. C) EMSA results summarized. Strong binding site (filled circle), moderate (grey circle), weak (open circle), debatable (dotted circle, fragment OH5) no binding observed (no circle). Reprinted from original article (I), copyright (2007), by permission of Oxford University Press.

The novel binding sites found *in vitro* for MTERF using EMSA were studied *in vivo* by means of semiquantitative mIP analysis modified from the one published by Lu *et al.* (2007). We were successful at amplifying the $tRNA^{Leu(UUR)}$ binding site containing fragment in the immunoprecipitate using anti-Myc antibody whereas it was not immunoprecipitated by anti-FLAG antibody or no antibody control reactions. Uninduced cells were used as control and generally the immunoprecipitates from those cells were negative in the given conditions, but sometimes when excess of anti-Myc antibody was used, a weak band was detected that correlated with the low level of leaky expression of

MTERF-MycHis fusion protein. Fragments that were found to bind MTERF *in vitro* based on the EMSA assay and chosen to be further studied by means of mIP, namely ND1.1, NCR1, NCR5, OH1 and OH5, gave positive signals also in the anti-Myc immunoprecipitation reactions from induced MTERF-MycHis cells, whereas control reactions with anti-FLAG antibody or with no antibody gave no such band. In EMSA, MTERF did not show binding affinity against e.g. ND3.4 which was therefore chosen as a negative control for the mIP. This fragment gave no band or only a very weak band was detected. (Figure 5.9)

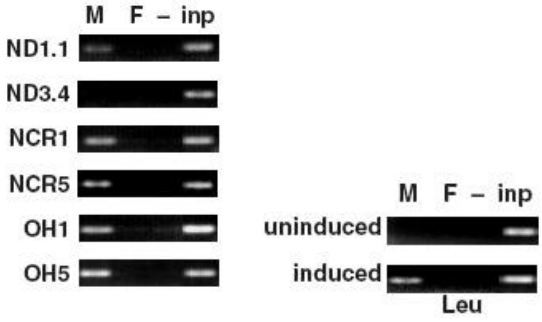

Figure 5.9. MTERF binding in human mitochondrial DNA *in vivo*. MTERF-MycHis binding in human mtDNA was studied by means of mIP. Immunoprecipitation was carried out using either anti-Myc (M), anti-FLAG (F) or no antibody (-) and the cells used were inducible Flp-In™ T-Rex™-293 cells over-expressing MTERF-MycHis. For Leu fragment non-induced cells were also tested. Reprinted from original article (I), copyright (2007), by permission of Oxford University Press.

5.1.8 MTERF binding motif (I)

As I had established that MTERF binds several sites in mtDNA, I then proceeded to study whether there is a sequence MTERF prefers to bind. I established SELEX (systematic evolution of ligands by exponential enrichment) to identify the consensus binding sequence for MTERF-MycHis. SELEX consists of several rounds of selecting and amplifying the most optimal fragments binding the protein of interest the results of which are shown in Table 5.1. When the SELEX consensus sequence was compared with the canonical MTERF binding site sequence within the $tRNA^{Leu(UUR)}$ coding gene the minimum binding sequence was found to be $GG(N_8)GG$. After 7 rounds of SELEX 82 clones out of 109 analysed contain a match to TGGT or TYGGT consensus sequence and

43 clones shared a consensus sequence TGGT(N_5)TYGGT or the complement thereof. (Table 5.1)

Table 5.1. SELEX results for the MTERF binding site analysis. Reprinted from original article (I), copyright (2007), by permission of Oxford University Press.

Position	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Occurrences[a]														
A	0	0	0	3	30	17	23	21	1	0	0	0	0	2
C	0	0	0	7	4	6	1	3	0	0	15	0	0	0
G	8	43	43	2	4	16	6	16	31	0	0	43	43	0
T	35	0	0	31	5	4	13	3	11	43	28	0	0	41
Consensus[b]	t	G	G	t	a	r	a	r	g	T	Y	G	G	t

[a]Out of 43 clones analysed which matched a clear consensus (see text).
[b]Nucleotides found in 43/43 clones shown in upper case, others in lower case, Y = pyrimidine, R = purine.

5.1.9 The effect of modifying MTERF levels on mtDNA copynumber (I)

One of the aims of my research was to study whether MTERF has a role in human mtDNA replication. Therefore, I first studied whether MTERF over-expression has any effect on mtDNA copynumber and whether it has novel binding sites in mtDNA in addition to the previously reported canonical binding site. Over-expression or silencing of MTERF did not affect the mtDNA copynumber (Figure 5.10, Figure 3c in III).

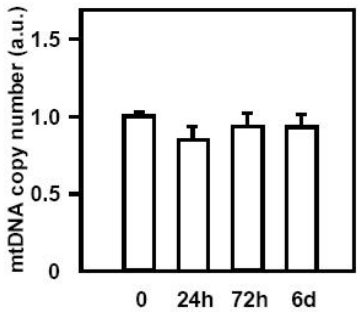

Figure 5.10 The effect of over-expressing MTERF on mtDNA copynumber. Quantitative PCR was used to analyze the mtDNA copynumber of Flp-In™ T-Rex™-293 cells transfected with the MTERF construct and induced for 24 h, 72 h or 6 d. Values were normalized to the mean value for uninduced cells. Reprinted from original article (I), copyright (2007), by permission of Oxford University Press.

5.1.10 Replication pausing at the canonical MTERF binding site (I)

Having found several novel binding sites for MTERF I wished to explore the possible biological significance thereof. One hypothesis was that some of the replication pause sites in the human mtDNA actually colocalise with MTERF binding sites. The MTERF binding site within the $tRNA^{Leu(UUR)}$ coding gene is known as the canonical binding site of MTERF and it was also the first region that was studied by means of 2DNAGE. mtDNA was isolated from different cell-lines and double digested with *Pvu*II and *Acc*I restriction enzymes in order to produce a 3.6 kb fragment covering the segment from O_L up until the rDNA (Figure 5.11A). Several pause sites were found and the precision of the autoradiographic signals was enhanced further by treating the mtDNA with S1 nuclease (Figure 5.12, 5.11B). Four cell lines were studied, namely HEK293T, HeLa, Jurkat (T-cell leukaemia) and 143B (osteosarcoma) cells, for the replication pause sites (Figure 5.12). Of these cell lines HEK293T and HeLa gave rather similar patterns, where pause sites could be clearly distinguished. First we were able to recognise a replication pause site (a) that maps to the location of $tRNA^{Leu(UUR)}$ coding gene, secondly a replication pause site (b) that maps to the 3' region of the ND1 gene or the IQM tRNA cluster next to it, thirdly a replication pause site (d) close to O_L and finally one within the ND2 coding gene (c). Jurkat and 143B cells gave rather similar patterns as the HEK293T and HeLa cell lines although there were generally less mtDNA replication intermediates in these two cell lines and S1 treatment did not enhance the separation of the pause sites in the gel. The replication pause site located in the ND2 coding gene could not be detected in Jurkat and 143B cells whereas the replication pause site at $tRNA^{Leu(UUR)}$ could be detected in all the cell lines studied (Figure 5.12).

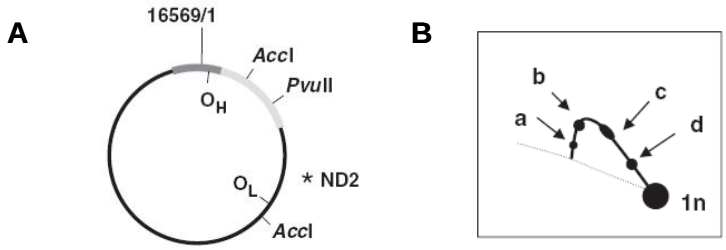

Figure 5.11. 2DNAGE for MTERF effects on replication pausing at region spanning from 16S rDNA till O_L. A) *Acc*I and *Pvu*II restriction sites shown in the map as well as O_H and O_L sites. Probe used was ND2, marked with asterisk. Non-coding region (NCR) enhanced bold, dark grey and rDNA is enhanced bold, pale grey. B) Schematic map of the prominent pause sites a, b, c and d. Reprinted from original article (I), copyright (2007), by permission of Oxford University Press.

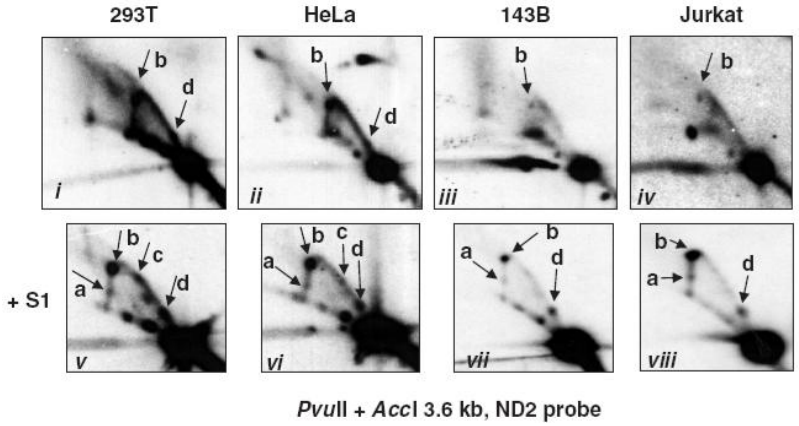

Figure 5.12. Mitochondrial replication is paused at the canonical MTERF binding site. Four human cell lines were assayed for *Pvu*II-*Acc*I fragment by means of 2DNAGE and probed with ND2 probe. S1 nuclease treated samples were compared to non treated ones. The prominent mtDNA replication pause sites a, b, c and d are denoted with arrows. Reprinted from original article (I), copyright (2007), by permission of Oxford University Press.

5.1.11 MTERF promotes mtDNA replication pausing (I)

Two approaches were applied to study whether MTERF promotes mtDNA replication pausing in human mtDNA, namely over-expressing MTERF in inducible cells and on the other hand knocking down MTERF by means of RNA interference using short interfering RNAs. mtDNA was extracted from cells over-expressing MTERF and double digested with *Pvu*II and *Acc*I and compared to similarly treated mtDNA from non-induced cells. The restriction sites and the binding sites of the probes are illustrated in Figure 5.13A.

The 3.6 kb fragment containing the canonical MTERF binding site was probed for and the pause site at $tRNA^{Leu(UUR)}$ (a) was observed to be strongly enhanced under condition of MTERF over-expression. Also the ND1/IQM tRNA cluster (b), O_L (d) and ND2 (c) pause sites were enhanced by MTERF over-expression. X-form intermediates (x) were also enhanced by MTERF over-expression (Figure 5.13B).

When the effect of over-expressing MTERF on the non-coding region was studied it was established that it enhances the paused replication intermediates migrating near the bubble arc (bubble arc denoted as 'f' in the *Hinc*II digested fragment and as 'n' in the equivalent *Acc*I fragment, probed with 'O_H' probe) (Figure 5.13C). Bubble arc consists of replication intermediates that contain a bubble. Once bubble exits the fragment studied the bubble disappears and converts into Y-shaped RIs. Over-expressing MTERF also decreased the amount of termination intermediates (t) (Figures 5.13C and 5.13D) and on the other hand generally enhanced the representation of Y-form replication intermediates (g) (Figure 5.13C).

Human mitochondrial DNA was mapped throughout by means of 2DNAGE to look for other potential effects caused by MTERF over-expression and it was observed that MTERF over-expression gave rise to a novel replication pause site at ND3 (Supplementary Figure 4C in I). However, EMSA did not show MTERF binding activity on this site (Supplementary Figure 2 of I). A prominent pause site (h) close to ND5/ND6 gene junction site was not affected by MTERF over-expression (5.13C).

The effect of MTERF over-expression on human mitochondrial DNA replication was studied further by means of 2DNAGE (mtDNA digested with a restriction enzyme that cuts only once in the genome). The findings supported the idea of enhanced pausing in the ND1/$tRNA^{Leu(UUR)}$ region. The effects observed in these gels caused by MTERF over-expression were site-specific, no general slowing down of replication, that is typical for non-specific stalling, was observed (Figure 5.13D, Supplementary Figures 4D and 4E in I).

When MTERF was knocked down by means of RNAi it inferred replication pausing was decreased in the ND1/ $tRNA^{Leu(UUR)}$ region (Figure 5.14.). When RNAi treated cells were compared to those not treated, the replication pause site (a) at $tRNA^{Leu(UUR)}$ disappeared and X-form replication intermediates were diminished. Other replication pause sites seemed to be affected to a lesser degree (Figure 5.14).

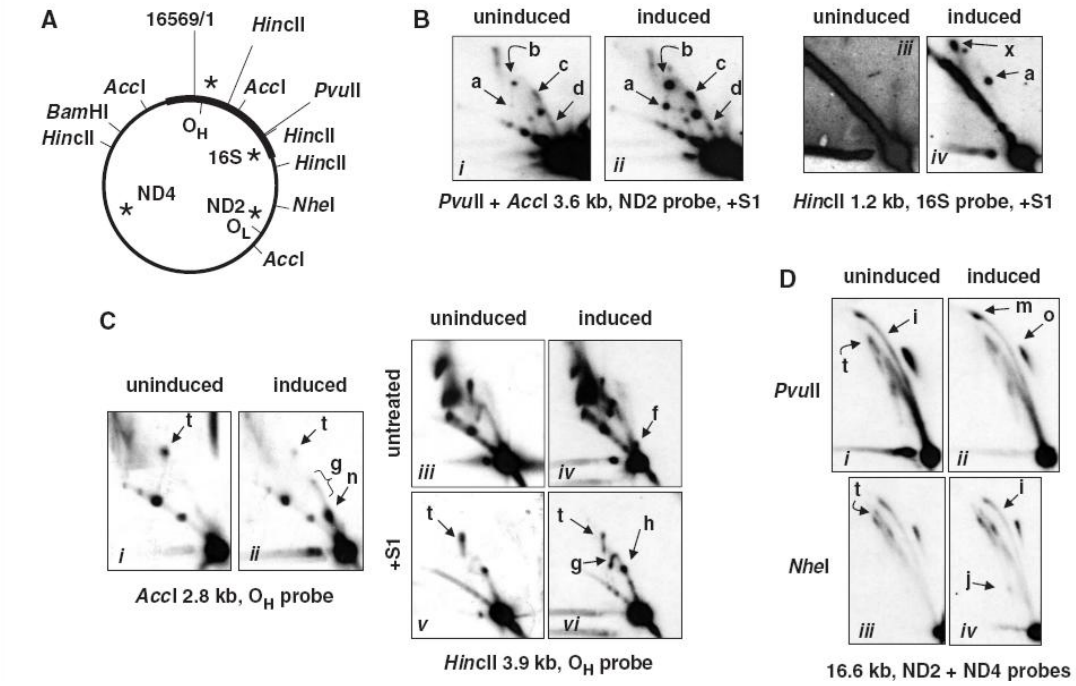

Figure 5.13. MTERF promotes mitochondrial replication pausing. Here cells induced to over-express MTERF are compared to non-induced ones. For clearer visualization of the pause sites the samples were treated with S1 nuclease where indicated. Pause sites a-d and other replication intermediates are denoted with arrows. A) Relevant restriction sites and locations of the probes illustrated in the map. B) Unit-length fragment signal was measured by phosphoimaging and the induced and uninduced blots were adjusted so as to be comparable. C) Pause site h was not affected when levels of MTERF were modulated whereas termination intermediates (t) were decreased and Y form replication intermediates (g) were enhanced. D) In single hitter gels paused bubble (m) and the double-Y species (j) were upregulated when MTERF was over-expressed. They both are predicted products yielded by replication pausing at the ND1/$tRNA^{Leu(UUR)}$ site. Also X, double-Y and broken theta molecules are affected and most clearly putative termination intermediates (t) are suppressed by MTERF over-expression. Uncut and gapped circles, denoted 'o', were diminished by MTERF over-expression in the *Pvu*II but not *Nhe*I digest. Reprinted from original article (I), copyright (2007), by permission of Oxford University Press.

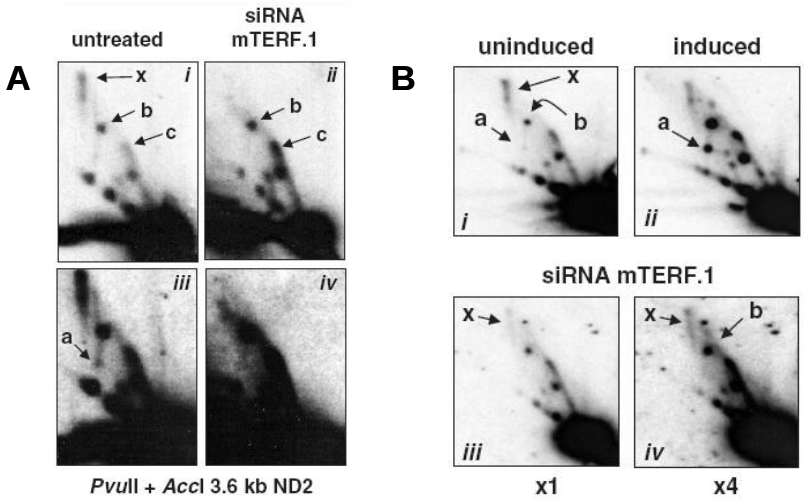

Figure 5.14. Effect of silencing MTERF on human mtDNA replication intermediates. A) siRNA MTERF.1 transfected cells (cells harvested 48 h after transfection) were compared to untreated HEK293T cells. *Pvu*II and *Acc*I digested samples were S1 treated and the 3.6 kb fragment was probed with ND2. Two exposures are shown for both, siRNA MTERF.1 transfected and the untreated cells. X-spike (x) and pause site 'a' are both suppressed by silencing MTERF and also pause site 'b' is downregulated when compared to pause site 'c'. B) Uninduced (i) and induced (ii) MTERF over-expressing cells (reproduction of figure 4B in I) shown alongside with MTERF silenced cells (iii). Silencing MTERF abolishes pause site 'a' as can be seen from longer exposure (iv). Reprinted from original article (I), copyright (2007), by permission of Oxford University Press.

5.2 Functional studies on MTERFD1 and MTERFD3 (III)

5.2.1 MTERFD1 and MTERFD3 are mitochondrial proteins (III)

For MTERFD1 and MTERFD3 studies I established inducible epitope-tagged fusion protein expressing cell lines using the Flp-In™ T-Rex™-293 cell system (HEK293T cells were also transiently transfected with the MTERFD3-FLAG expression construct). The expression of the epitope-tagged variants of MTERFD1 and MTERFD3 was verified by means of Western blotting. The size of the MTERFD3-HA or MTERFD3-FLAG protein was as predicted, roughly 43 kDa, when the epitope tag size has been taken into account and the mitochondrial targeting sequence has been removed. MTERFD1-FLAG was observed to migrate slightly slower than expected, approximately 47 kDa (Figure 5.15A).

The intracellular localization of the fusion proteins was studied by means of immunocytochemistry of transiently transfected cells. Both FLAG epitope-tagged proteins were mitochondrially targeted *in vivo* based on colocalization with Mitotracker Red. MTERFD1-FLAG but not MTERFD3-FLAG showed a distinctive punctuate staining pattern that suggested it is localized in nucleoids (Figure 5.15B).

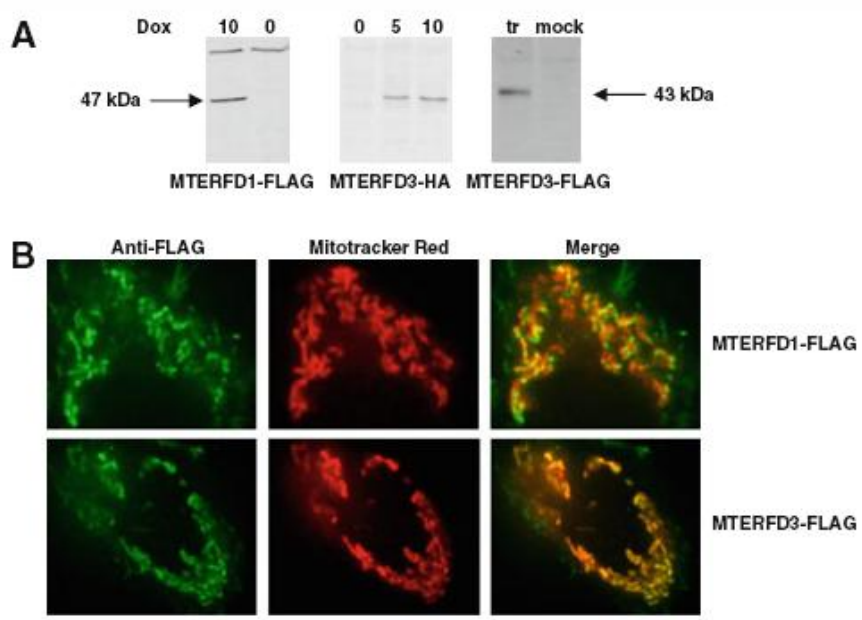

Figure 5.15. Epitope tagged MTERFD1 and MTERFD3 are efficiently over-expressed in human cells and mitochondrially targeted. A) Flp-In™ T-Rex™-293 cells were doxycycline induced for 48 h to express either epitope tagged MTERFD1-FLAG or MTERFD3-HA. HEK293T cells were transiently transfected (tr) with MTERFD3-FLAG and compared to mock transfected. Western blots using total protein extracts were probed with anti-FLAG or anti-HA antibodies. B) HEK293T cells were transiently transfected with MTERFD1-FLAG or MTERFD3-FLAG for immunocytochemistry and 24 h after transfection probed with anti-FLAG antibody and counterstained with Mitotracker Red. Merge figure (created using false colours) shows that the stains colocalise. Reprinted from original article (III), copyright (2010), by permission of Springer.

5.2.2 MTERFD1 and MTERFD3 do not bind mtDNA sequence specifically (III)

SELEX and EMSA were used to study whether MTERFD1 and MTERFD3 bind DNA in sequence-specific manner. Previously I used SELEX successfully to look for the

preferred binding motif for MTERF-MycHis. The same conditions were used to look for binding motifs for MTERFD1-FLAG and MTERFD3-HA. However, SELEX results were negative in both cases after 7 rounds of selection. Two independent experiments were carried out for both proteins. 69 and 15 sequences were obtained for MTERFD1-FLAG whereas 25 and 29 clones were sequenced for MTERFD3-HA, and these sequences did not differ from the negative controls (35 clones sequenced).

A series of EMSA assays was carried out using mitochondrial extracts from cells transiently transfected with either MTERFD1 or MTERFD3 or from induced MTERFD1-FLAG or MTERFD3-HA Flp-In™ T-Rex™-293 cells and using the same probes as was used for MTERF-MycHis EMSAs. The whole non-coding region (NCR) was checked for MTERFD1-FLAG and MTERFD3-HA binding as well as the canonical MTERF binding site at the 16S/tRNA$^{Leu(UUR)}$/ND1 gene junction region, the IQM tRNA cluster together with the C-terminal portion of ND1, O_L and the ATPase6/COIII, ND5/ND6 and tRNA^{Pro}/tRNA^{Thr} gene junction sites that are all fragments where MTERF and its homologues or other mtDNA binding proteins have previously been reported or suggested to bind. However, there was no sign of mtDNA binding activity for either of the proteins studied, since no novel protein-DNA complexes were observed nor any clear changes in mobility were visible. Also the effect of over-expressing MTERFD1-FLAG or MTERFD3-HA on MTERF-MycHis binding was studied but no novel or enhanced bands compared to control cells were observed. When supershift assays are considered there were no any changes in mobility of the complexes in MTERFD1-FLAG over-expressing cells whereas cells over-expressing MTERFD3-HA did yield a supershift with anti-HA antibody when probed with 4 fragments of the promoter region or with ATPase6/COIII gene junction site covering fragment. Thus, although neither EMSA nor SELEX revealed plausible binding sites for either MTERFD1 or MTERFD3, MTERFD3 may associate with DNA-binding complexes in some specific regions of the genome. My findings indicate therefore that these proteins do not directly bind mtDNA but might be able to do so by interacting with other proteins (Figure 5.16).

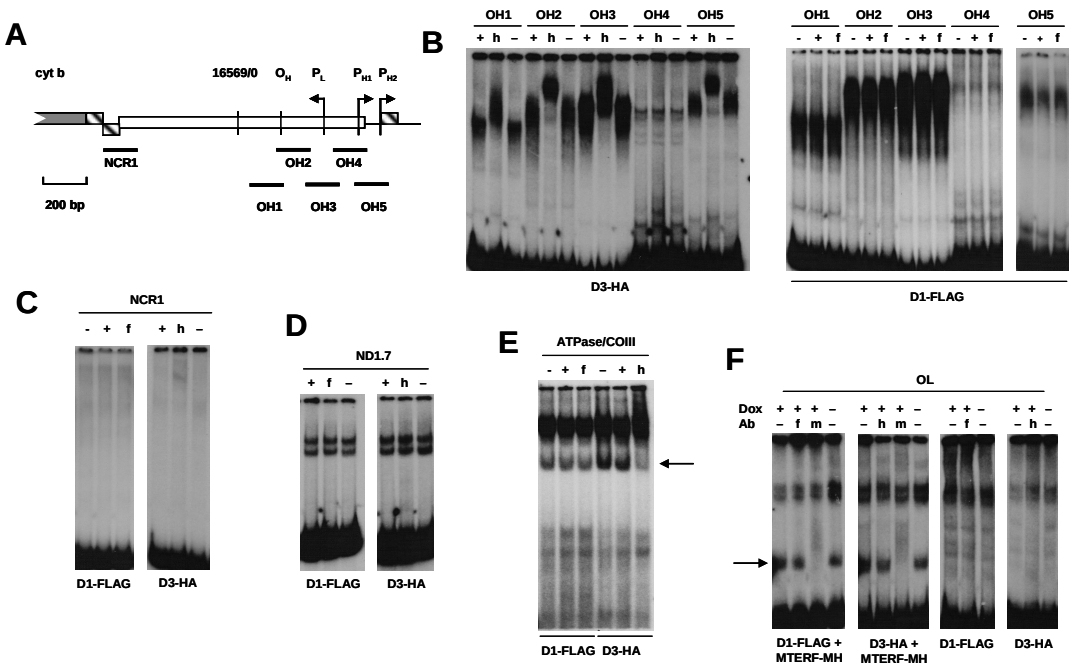

Figure 5.16. MTERFD1 and MTERFD3 binding in mitochondrial DNA *in vitro*. A) 150 bp long probes used in B) as illustrated. Promoters are denoted as P_L, P_{H1} and P_{H2}. O_H is the heavy strand replication origin and terminus; tRNA coding genes denoted with hatched bars, upper and lower bars for encoded genes on heavy and light strand, respectively, protein coding gene denoted with grey bar. B)-F) Series of EMSA gels using crude mitochondrial protein extracts from Flp-In™ T-Rex™-293 cells transfected with MTERFD1-FLAG (D1-FLAG) or MTERFD3-HA (D3-HA) +/- doxycycline induced expression of protein of interest or MTERF-MycHis stably transfected cells (MTERF-MH) and EMSA probes as indicated. Supershift assay was carried out using the following antibodies: anti-FLAG (f), anti-HA (h) and anti-Myc (m). E) Complex formed by ATPase6/COIII oligonucleotide probe (denoted with an arrow) is modestly enhanced by MTERFD3-HA over-expression and partially supershifted by antibody. F) Arrow indicates the complex formed by MTERF-MycHis alone. Reprinted from original article (III), copyright (2010), by permission of Springer.

5.2.3 MTERFD1 and MTERFD3 downregulate mtDNA copynumber (III)

To investigate whether MTERFD1 and MTERFD3 have any role in mtDNA replication given the preceding results on MTERF, I tested whether altering the expression levels thereof affects the mtDNA copynumber. The effect of over-expressing MTERFD1-FLAG and MTERFD3-HA was studied over 7 days. MTERFD1-FLAG expressing cells showed progressive downregulation of the mtDNA copynumber which was statistically significant on day 7 (Figure 5.17A). On the other hand the induction of MTERFD3-HA

gave only a transient decrease of the mtDNA copynumber, at the boundaries of statistical significance (Figure 5.17B). When the proteins were knocked down in HEK293T cells there was no effect on mtDNA copynumber (Figure 5.17C).

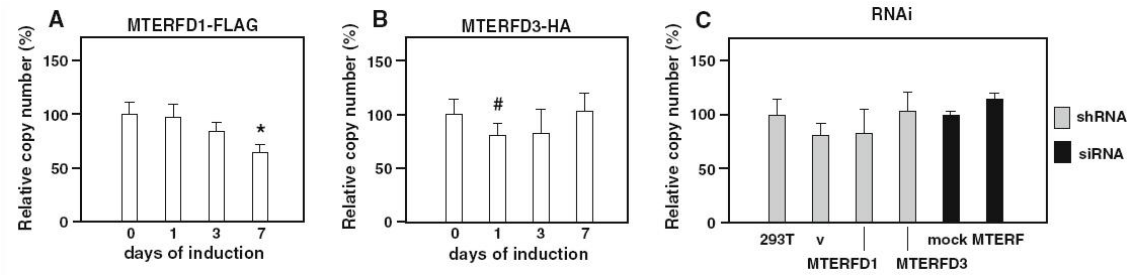

Figure 5.17. Effect of modulating MTERFD1 and MTERFD3 levels on mitochondrial DNA copynumber. A) Relative mtDNA copynumbers measured by means of Q-PCR for Flp-In™ T-Rex™-293 cells over-expressing MTERFD1-FLAG or B) MTERFD3-HA. Normalised against copynumber of the uninduced cells. C) Grey bars show HEK293T cells silenced either for MTERFD1 or MTERFD3, not treated or transfected with empty shRNA vector (v). Black bars show MTERF-silenced cells (using siRNAs) in comparison with mock transfected cells. Data was normalized against relevant untransfected or mock-transfected cells. Here statistical significance is marked with * ($p < 0.01$) and borderline significance is denoted with # ($p = 0.05$, t-test). Reprinted from original article (III), copyright (2010), by permission of Springer.

5.2.4 MTERFD1 and MTERFD3 influence mtDNA replication intermediates (III)

To study the role of MTERFD1 and MTERFD3 proteins in mtDNA replication we used 2DNAGE to study the effect of over-expressing MTERFD1-FLAG or MTERFD3-HA in Flp-In™ T-REx™-293 cells compared to controls. Over-expression of either MTERFD1-FLAG or MTERFD3-HA produced a number of changes that indicated that over-expression of either leads to impaired completion of replication. The over-expression of MTERFD3-HA had a stronger effect than that of MTERFD1-FLAG (Figure 5.18A).

For this assay mtDNA was digested with *Pvu*II, which cuts mtDNA once, about 2.5 kb upstream of the replication terminus near O_H. Schematic drawings of the replication intermediates are shown in Figure 5.18C. Multi-junctional molecules, interpreted as double Y intermediates, were observed to be upregulated, indicating that over-expression of either of the proteins has effect on the final steps of replication. When MTERFD3 was

over-expressed uncut or gapped circles (gc), that are released by the resolution step, were depleted. Lagging-strand DNA synthesis remains unfinished in the uncut circles at the *Pvu*II restriction site. The bubble arc, represents an intermediate produced at an early stage of replication, was unchanged (Figure 5.18A).

The effect of over-expressing MTERFD1 and MTERFD3 in mtDNA replication intermediates was almost opposite to that of over-expressing MTERF. Over-expression of MTERF led (Figure 5.18A) to accumulation of bubble intermediates that are paused near the high-affinity MTERF binding site. Conversely both multi-junctional structures and gapped circles were depleted by MTERF over-expression. When either MTERFD1 or MTERFD3 was knocked down by means of RNAi, most of the mtRIs were preserved, but incomplete circles accumulated (Figure 5.18B).

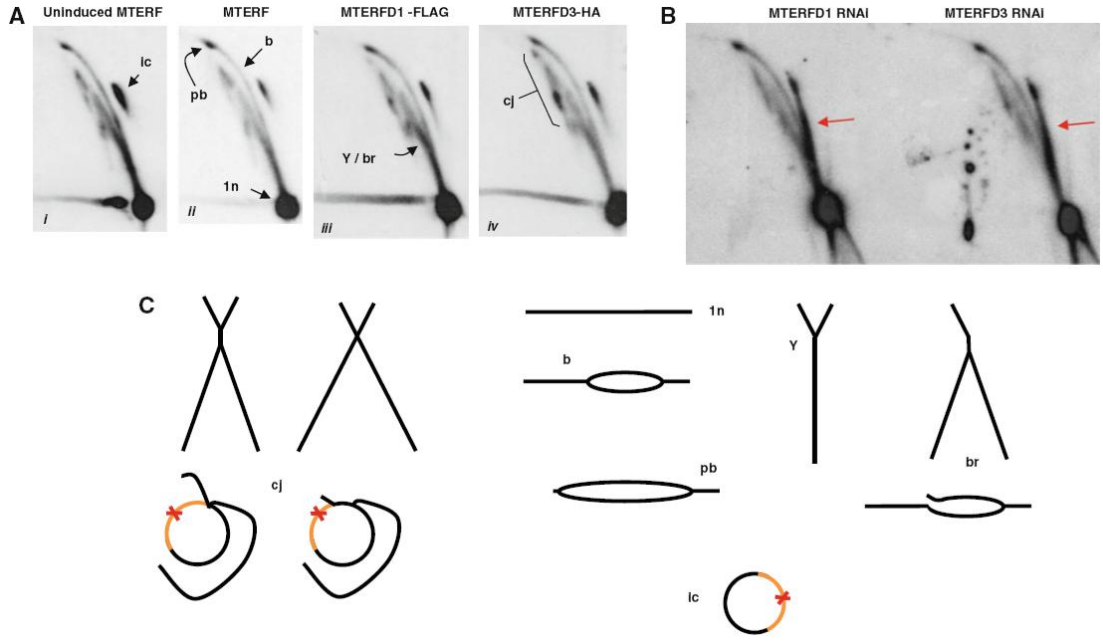

Figure 5.18. The role of MTERFD1 and MTERFD3 in mitochondrial replication. A), B) 1 µg of total nucleic acids, digested with PvuII and probed with (ND2 + ND4) probe, were analysed by 2DNAGE. Cells were harvested 48 h after induction with 10 ng/ml doxycycline or after transfection with shRNA constructs. Panels *i* and *ii* are reproductions of Figure 4D in I. Replication intermediates are denoted with arrows and schematic drawings are shown in C), bubble arc (b), incomplete circles (ic), Y species (Y), broken replication intermediates (br), complex junctional forms (cj). Red arrows in B) indicate incomplete circles with extensive single-strandedness. C) Schematic drawings of the mitochondrial replication intermediates. Uncut *Pvu*II site is marked with red cross. Reprinted from original article (III), copyright (2010), by permission of Springer.

5.3 Functional studies on the mitochondrial transcription factor A (IV)

5.3.1 The effect of altering TFAM levels on mitochondrial transcripts (IV)

One of the aims was to study how modulating TFAM levels in cultured human cells affects mitochondrial tRNA transcript levels compared to modulating mTERF levels. Another aim was to compare the effects of altering TFAM and MTERF levels on mtDNA replication. I contributed to article IV by studying the effect of modulating TFAM levels on mitochondrial transcripts by means of neutral acrylamide/urea gel electrophoresis, in collaboration with Jaakko Pohjoismäki.

The steady-state level of mitochondrial transcript ND3 mRNA (normalized to S5 rRNA) was studied in Flp-In™ T-Rex™-293 cells stably transfected with either TFAM-stop or TFAM-MycHis induced to over-express *in vivo*. Over-expression of TFAM or TFAM-MycHis induced a clear decrease in the level of ND3 mRNA (Supplementary Figures 1D and 2D in IV).

We studied also the effect of over-expressing TFAM-MycHis on tRNA levels during the recovery period after depleting the mtRNA and mtDNA by three days of EtBr treatment. Over-expressing TFAM markedly slowed down the recovery of the tRNA levels after the removal of the drug compared to cells not induced to over-express TFAM (Figure 5.19). The result obtained here contrasts with the minimal effect seen when MTERF was over-expressed (Figure 5.5C).

Western blotting showed that knocking down TFAM by means of RNAi lead to progressive reduction of TFAM protein levels and mtDNA copynumber (Supplementary Figure 3 in IV). When the cells were treated with TFAM specific siRNAs up to 48 h, no clear effect on the TFAM protein, mtDNA or ND3 mRNA transcript levels (normalized to the amount of template mtDNA) were detected (Figure 1 in IV).

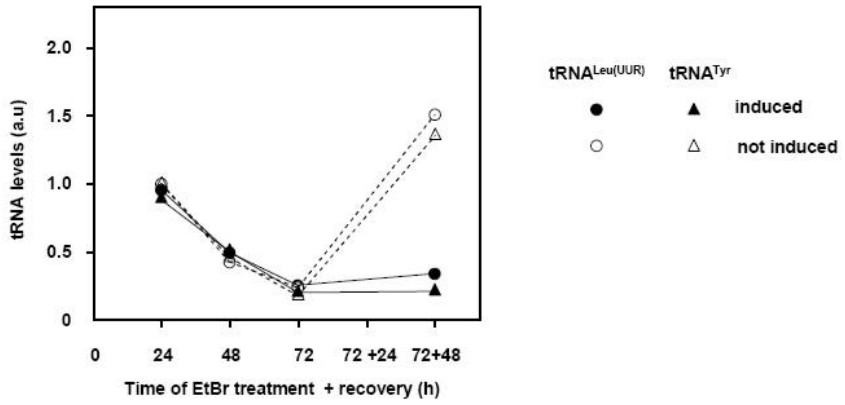

Figure 5.19. The effect of over-expressing TFAM-MycHis on mitochondrial tRNA levels. Cells induced to over-express TFAM-MycHis were treated with EtBr for 72 h in parallel with non-induced cells after which EtBr was washed away. Mitochondrial tRNA levels were studied during the five days of induction of TFAM expression compared to non-induced cells by means of Northern blot and normalized with 5S rRNA. Reprinted from original article (IV), copyright (2006), by permission of Oxford University Press.

5.3.2 TFAM is a regulator of the mode of mitochondrial DNA replication (IV)

The effect of modifying the levels of TFAM expression on mtDNA maintenance were studied in cultured human cells. By means of real-time PCR and Southern blot it was shown that TFAM-stop over-expression decreased the mtDNA copynumber by 40-60% (Supplementary Figure 1 in IV) even if the TFAM protein levels did not increase markedly (Supplementary Figure 1 in IV). Knockdown of TFAM by means of RNAi also resulted in progressive decrease in mtDNA copynumber (Figure 1 and Supplementary Figure 3 in IV).

The effect on mitochondrial DNA replication intermediates of over-expressing TFAM-stop or TFAM-MycHis was studied, which gave a clear overall increase in the amount of RIs relative to the unit-length restriction fragment (Figure 2 and Supplementary Figure 5 in IV). When DNA from cells induced to over-express TFAM was digested with *Pvu*II, an increase in the clubheaded bubble arc (representing

unidirectional theta replication initiating at OH) was observed and a more prominent simple Y-arc than in uninduced control cells was detected.

In other digests, conducted to analyse shorter, specific fragments, we observed that S1 nuclease-sensitive RIs were decreased, whereas nuclease-resistant arcs were enhanced by TFAM over-expression (Figure 2c-e in IV). The strongest effects were observed in the region spanning from O_L through the rDNA (i.e. the so-called minor arc of the genome). In TFAM over-expressing cells complete Y-arcs were detected already at low exposure compared to uninduced cells, and the diffuse 'clouds' of nuclease-sensitive material were replaced by two or several nuclease-sensitive slow-migrating Y-shaped arcs.

RIs detected by probes for the NCR were not dramatically affected by TFAM over-expression, the termination region being the most affected. In this region, the bubble and X-arcs were diminished, whereas the descending portion of the Y-arc was strongly increased (Figure 2c in IV). TFAM over-expression also increased replication pausing at certain pause sites, namely within the ND1 gene, at O_L (Figure 3d in IV), within ND5 (Figure 2c in IV) and finally at many weaker pause sites, e.g. in ND4, ND3, COXIII, A6, A8 and COXII (Figure 3c in IV).

Silencing of TFAM enhanced ribonuclease-sensitive material in the rDNA region and its mobility was also affected (Figure 4c in IV). Silencing of TFAM also strongly upregulated the X-arc in the O_H –containing fragment (Figure 4b in IV).

6. Discussion

6.1 The role of MTERF in mitochondrial transcription termination

6.1.1 Effects of modulating MTERF levels on sense transcripts

One aim of the project was to study the effect of modulating the MTERF level on the relative activities of the human mitochondrial heavy-strand transcription units by measuring the relative levels of different mitochondrial transcripts under different conditions. I did this by means of Northern blot analysis and Q-RT-PCR using cells either over-expressing MTERF or cells knocked down for MTERF expression.

I studied the effect of over-expressing MTERF on the steady-state $tRNA^{Phe}/tRNA^{Leu(UUR)}$ ratio and established that there is no significant difference in it when studying different cell clones over-expressing the natural variant of MTERF compared to the control cells (Figure 5.5A). I also studied the effect of over-expressing MTERF on the 16S and ND1 transcript levels in Flp-In™ T-Rex™-293 cells stably transfected with the MTERF-MycHis construct induced to over-express MTERF compared to non-induced cells and observed no effect on the relative quantities of the transcripts in question (Figure 5.5B). Neither did I observe any effect on mitochondrial RNA levels during the recovery period after EtBr depletion of mitochondrial RNA and DNA, when cells over-expressing MTERF were studied (Figure 5.5C).

As an opposite approach to MTERF over-expression I studied the effects of silencing MTERF by means of RNAi on different mitochondrial transcript levels. When the effect of silencing MTERF was studied 7 days after the initial RNAi treatment there was a

modest drop detected in the $tRNA^{Phe}$/$tRNA^{Leu(UUR)}$ ratio in HEK293T cells, with a slight overall increase in mitochondrial transcript levels. Once again, no changes were observed in similarly treated MTERF-MycHis over-expressing cells (Figure 5.6A). Knocking down MTERF did not bring about any detectable effects on mitochondrial RNA levels during the recovery period after EtBr depletion of mitochondrial RNA and DNA (Figure 5.6B).

However, my research produced two pieces of evidence supporting MTERF having a role in the transcription of the mitochondrial heavy strand. Firstly, after MTERF knock down in normal cells there was a small increase in the amount of the two mitochondrial tRNAs representing each of the heavy strand transcription units to be detected, relative to cytosolic 5S rRNA (Figure 5.6A). Secondly, the levels of sense-strand 12S rRNA relative to 16S or 18S rRNA gene transcripts, measured by means of Q-RT-PCR, was markedly increased when MTERF was knocked down (Figure 5.7F). As sense-strand 16S and ND1 transcript levels relative to each other or to 18S were not significantly affected, I suggest that there exists a compensatory mechanism (Figures 5.7F and S3C in II). According this suggestion, knocking down MTERF sends out a signal for globally upregulated mitochondrial transcription or decreased turnover, in order to overcome the potential impairment in 16S rRNA biogenesis. The degree of MTERF knockdown that was reached in these experiments was only 50%, for which reason the effects of the knockdown may be underestimated. It is likely that in these experiments up to half of the cells still express MTERF normally and in the remaining cells expression is significantly decreased.

I conclude here that the amount of MTERF in cells does not regulate the relative steady-state levels of transcripts representing the two heavy-strand transcription units in a simple manner, but it seems to be modified by compensatory mechanisms. Previous studies carried out to study the role of MTERF in transcription termination have yielded diverse and seemingly contradictory results. Martin *et al*. (2005) and Asin-Cayuela *et al*. (2004) reported that MTERF stimulates transcription *in vitro* from PH1 in a rather crude system. When purified recombinant proteins (Asin-Cayuela *et al*. 2005) or crude extracts

with DNA-affinity purified MTERF were used (Asin-Cayuela *et al*. 2004), MTERF was not found to stimulate transcription. Chomyn *et al*. (1992) studied the effect of the A3243G mutation on mitochondrial transcript levels and the possible role of MTERF in the pathogenesis of MELAS syndrome and reported impaired mitochondrial protein synthesis, defects in respiration and decreased binding affinity of purified MTERF to its target sequence due to the A3243G mutation; however, no major changes in mitochondrial rRNA or mRNA levels were observed. Fernandez-Silva *et al*. (1997) established *in vitro* that MTERF alone cannot bring about transcription termination. Shang and Clayton (1994) studied whether the A3243G mutation impairs transcription termination *in vivo* but found no evidence for this even though the mutation caused a drop in the rate of transcription termination *in vitro*, supposedly by reducing the MTERF binding affinity to its target site.

Previously Selwood *et al*. (2000) reported that TAP (thiamphenicol) treatment does not affect 12S and 16S rRNA levels in HepG2 cells but instead increases the steady-state levels of both mRNAs and tRNAs transcribed from the I_{H2} transcription initiation site of the heavy strand. Thiamphenicol is an antibiotic which blocks mitochondrial protein synthesis. Their findings indicate that modulation of MTERF complex could be the limiting factor determining the mitochondrial gene expression at the level of transcription termination. The underlying mechanism was proposed to be TAP enhancing transcriptional initiation from the second HSP (I_{H2}) transcription initiation site, which was first suggested by Montoya *et al.* (1983). This would explain the unchanged rRNA levels and also the enhanced transcription downstream of the MTERF binding site.

Findings from the study of thyroid hormone action (Enriquez *et al*. 1999) and of the effects of variation in ATP supply (Micol *et al*. 1997) are both in agreement with our results. The molecular mechanism behind suggested is that they have an effect on the relative rates of transcription of the two differentially transcribed transcription units of the mitochondrial heavy-strand but no effect on that at the high affinity MTERF binding site.

Some of the MTERF homologues in other organisms have been shown to affect mitochondrial transcription which argues against the idea that the effects on nucleic acid metabolism caused by altering MTERF levels are incidental while the real biological function of MTERF is still lurking somewhere else inside mitochondria. The *Arabidopsis thaliana* gene *SOLDAT10* (singlet oxygen-linked death activator) encodes a protein that is related to the human MTERF protein. The *soldat10* mutation has been reported specifically to decrease plastid rRNAs which indicates that this mutation does not universally impair chloroplast RNA accumulation (Meskauskiene *et al*. 2009). The *soldat10* mutation has been also suggested to have more indirect effect on the ROS production or the redox state of the plastid. This mutation leads to disturbance of plastid homeostasis which suppresses ROS-mediated cell death (Meskauskiene *et al*. 2009). The MTERF protein homologue in *Chlamydomonas reinhardtii*, MOC1, on the other hand, has a role in restoring mitochondrial RNA levels after exposure to light (Schönfeld *et al*. 2004). MOC1 levels are upregulated after light exposure and the loss of MOC1 leads to light-sensitive phenotypes as well as impairing the chloroplast transcription and replication (Schönfeld *et al*. 2004).

6.1.2 MTERF regulates the levels of anti-sense transcript levels in human mtDNA

In this project I addressed the question whether MTERF could have a limiting role in transcription termination in human mitochondrial DNA *in vivo*. By modulating MTERF levels and studying the effect on sense and antisense 16S and ND1 transcripts, I found out that over-expressing MTERF decreases the anti-16S/anti-ND1 transcript ratio and knocking down MTERF increases it. I therefore established that MTERF regulates antisense transcript levels consistent with it facilitating of antisense transcription termination initiated at PL by binding to its canonical binding site. The effect of modifying MTERF levels on RNA19 (16S + ND1) remains to be studied. It should be interesting to do so, as it could shed more light on the antisense results.

Why would MTERF have a role in regulating mitochondrial antisense transcripts that have no documented function, and which are assumed to be destined for turnover? Asin-Cayuela *et al.* (2005) showed, using recombinant human MTERF in a highly purified reconstituted *in vitro* transcription system, that MTERF transcription termination exhibits clear polarity. When bound in the 'forward' orientation to its target sequence in the heavy strand promoter, MTERF arrested transcription completely, but when bound in the opposite orientation, it arrested transcription only partially. Asin-Cayuela *et al.* (2005) also showed that MTERF alone can terminate transcription *in vitro* and does not need to be post-translationally modified by phosphorylation. It might be that MTERF actually is capable of terminating transcription in a bidirectional manner also *in vivo* and the effects on the sense transcript levels are masked or complicated due to the post-transcriptional processing of rRNAs, the stabilization of rRNA into ribosomal subunits or due to compensatory effects on transcriptional initiation or RNA stability.

The biological significance of the antisense transcripts is still unclear but one possibility is that they could have a role in providing a stalled replication fork with a primer that is needed for the re-initiation of the replication machinery. Non-coding antisense RNA transcripts have been found in normal proliferating cells arising from the 16S rRNA gene, whereas in tumour cell lines the levels of these antisense transcripts are downregulated (Villegas *et al*. 2007, Burzio *et al*. 2009). It therefore seems that the antisense transcript levels may relate to the tumorigenicity or proliferation status of the cell. The exact physiological origin of these antisense transcripts is still unknown but they might be created post-transcriptionally or during transcription by template strand-switching.

My results suggest that MTERF has a role in mitochondrial transcription *in vivo*, but it does not seem to set the levels of mature mitochondrial transcripts encoded by the PH1 and PH2 heavy-strand transcription units in a simple manner. Rather, this is influenced by compensatory mechanisms.

6.2 MTERF binding to human mtDNA

Previously, MTERF has been reported to have two binding sites in the human mitochondrial DNA. The first is canonical binding site, located downstream of the mitochondrial rRNA coding sequences within the $tRNA^{Leu(UUR)}$ gene. A 13 bp fragment within the MTERF binding site has been reported to be crucial to terminate transcription correctly from PH1 *in vitro* suggesting that it may be needed for the formation of the 3' end of 16S rRNA (Christianson and Clayton 1988). The ability of the MTERF binding site to work in a bidirectional manner has also been shown to be crucial for accurate termination of transcription (Christianson and Clayton 1986).

As I was interested in the possible roles of MTERF in human mitochondrial replication, I first studied whether MTERF has other binding sites in mtDNA than the previously reported canonical binding site within the $tRNA^{Leu(UUR)}$ (Kruse *et al.* 1989) coding gene and the other one at the PH1 initiation site for the H strand transcription, more recently reported by Martin *et al*. (2005). There are various examples of MTERF homologues binding to multiple target sites within mtDNA. The MTERF homologue in sea urchins, mtDBP, binds sea urchin mtDNA at two sites that are located opposite each other in the circular genome. The MTERF homologue in *Drosophila melanogaster*, DmTTF, also has two binding sites in *Drosophila* mtDNA, both of them located in non-coding DNA.

The novel binding sites that I found for MTERF, in human mtDNA both *in vitro* and *in vivo*, did not exhibit as high affinity binding as the earlier reported canonical MTERF binding site. However, these novel binding sites appeared to be clustered in the human mitochondrial genome indicating that MTERF might bind adjacent target sites in multiple copies to bring about a physiological effect. Since I used crude mitochondrial protein extracts for the EMSA experiments, it is also possible that MTERF co-operates with some other protein e.g. TFAM or one of the novel MTERF protein family members to bring about more efficient binding. It is also possible that post-translational modification, e.g. phosphorylation, is needed for MTERF to be biologically active.

MTERF binds mtDNA as a monomer and it is active in transcription termination *in vitro* only when present as a monomer (Asin-Cayuela *et al*. 2004). The latter authors further proposed that MTERF is present in mitochondria in two forms, an active monomer and an inactive homotrimer and that the relative amount of the active MTERF is regulated by the transition between the two forms. It should be interesting to measure more carefully the exact MTERF levels in different cell lines and tissues, and determine the exact nature of these complexes.

It should also be noted that MTERF could be over-expressed at such high excess in the cell lines I used for my studies that the vast excess of the protein binds mtDNA unspecifically all around the genome, but that only binding to the canonical site has any physiologically meaningful effect, whether on transcription or replication. This would of course require that there is not already an excess of MTERF protein in cells. Clearly further studies are needed here.

6.3 The physiological significance of multiple MTERF binding sites in the mtDNA

By means of SELEX I established that the minimum requirement for MTERF binding is GG(N_8)GG. When human mtDNA is studied, there are actually clusters of such minimum requirement binding sites found. Interestingly there are 12 such sites in the NCR alone, all in the same orientation. Christianson and Clayton (1986) previously reported possible binding sites within the NCR for MTERF. There is also a cluster of seven similar binding sites, again all in the same orientation, near the 3’ end of the ND1 coding sequence, spanning the IQM tRNA gene cluster adjacent to it. Curiously, the sequence at the canonical binding site shows the opposite orientation and overlaps on each side two other similarly oriented minimal MTERF binding sequences. Orientation-dependent termination activity has later suggested to be due to MTERF preferring to bind to the light strand of the mitochondrial DNA (Nam and Kang 2005). Clearly, MTERF binding

sites are not in random orientation or location in the mtDNA (Supplementary Figure 6 in I).

Prieto-Martin *et al*. (2004b) reported that the rat MTERF protein binds the mitochondrial transcription initiation site but did not detect any complex bringing together the rat mtDNA transcription initiation and termination sites and the rat MTERF. Martinez-Azorin (2005) had already introduced the idea that regulation of different mitochondrial transcripts is brought about by looping of mitochondrial DNA, when Martin *et al*. (2005) published a report of MTERF binding simultaneously at two sites in the mtDNA and therefore creating a loop in the mtDNA bringing together the transcription initiation and termination sites. According to Martin *et al*. (2005) this could explain the difference between the synthesis rates of mitochondrial rRNAs and mRNAs; namely, that rRNA is synthesized 15-60 times more than mRNA. However, my findings do not provide any support for this model *in vivo*, although one has to keep in mind that I did not measure synthesis.

6.4 Physiological significance of MTERF levels

The physiological amount of MTERF molecules present in mitochondria remains to be determined. There might, for example, be a large pool of inactive MTERF trimers present (Asin-Cayuela *et al*. 2004). If this were the case, over-expressed MTERF such as in my experiments would simply end up adding further to the inactive pool of MTERF. On the other hand, RNAi may not lead to detectable effects since a substantial or at least adequate pool of MTERF may remain; thus my negative results could also be explained away. Since all the cells used in this study are derived from one cancer cell line the possibility must also be considered, that our cells do not behave in a physiologically similar manner as non-cancer cells and might already have a higher level of MTERF. Therefore, altering MTERF levels could have a more profound effect on mitochondrial transcription in some other cell-types. Gene expression data from biogps.gnf.org does not support this idea, but shows that rather MTERF is expressed in HEK293T cells at very

close to the median level of all cell lines studied. The range of expression according to biogps.gnf.org is approximately 2-5 fold, whereas I achieved greater than 10-fold expression changes in my cells which is probably higher than in any normal physiological situation and therefore should be sufficient to yield detectable effect, if any could be produced.

It is also possible that MTERF is constitutively inactivated in HEK293T cells no matter what the expression level is. For example Prieto-Martin *et al*. (2004a) established that in order to be active in transcription termination *in vitro* rat MTERF needs to be phosphorylated. Asin-Cayuela *et al*. (2005) on the other hand reported that human recombinant MTERF does not need to be post-translationally phosphorylated, in order to carry out transcription termination *in vitro*. Therefore it would be interesting to check if over-expressed human MTERF is phosphorylated (or modified in some other way), whether it trimerizes or is held in complexes with other proteins or, indeed, whether it is cleaved to the active form.

6.5 A possible role of MTERF in mitochondrial DNA replication

6.5.1 MTERF has a dual role in transcription and replication arrest

One aim of my project was to establish whether MTERF has another role(s) in mtDNA maintenance relating to its novel binding sites. I addressed the question by studying the effects of MTERF in mtDNA replication by means of 2DNAGE. The outcome of the study was that, when over-expressed in inducible T-Rex cells, MTERF was demonstrated to promote replication pausing at several sites. In addition I documented novel replication pause sites in human mtDNA.

My results indicate that MTERF does not directly regulate the relative rates of PH1 and PH2 transcription. Since the different quantities of mRNA and rRNA transcripts in

human mitochondria can be explained based on their different half-lives and RNA processing efficiency, and considering also that the A3243G MELAS mutation does not affect mtRNA levels *in vivo*, it is thus possible that MTERF has another principal role in mitochondria than transcriptional regulation as such. My findings suggest that MTERF might have a dual role in transcription and replication arrest and may even coregulate these processes.

The light strand promoter (LSP) has been suggested to provide RNA primers for DNA replication because it is located near to the origin of heavy-strand replication. Light strand transcripts initiating from the non-coding region were initially found by Wallberg and Clayton (1983). Later it was established that the LSP transcription unit provides replication with RNA primers (Chang and Clayton 1985). The primer for the initiation of mtDNA replication is proposed to be synthesized by transcription from PL, but with the transcript remaining annealed to the template-strand. Yasukawa *et al*. (2006) reported results indicating that the vertebrate mtDNA lagging strand is first copied as extended RNA segments which are then matured to DNA. The lagging-strand priming described above is hypothesized to require either a primase that can synthesize extended RNA primers or alternatively it can happen via the so-called bootlace model. In the bootlace model the preformed L-strand RNA is hybridized with the displaced H-strand in a 3´ to 5´ direction as the replication fork proceeds. The bootlace model is supported by the MTERF enhanced replication pausing. By catalyzing a coincidental arrest of both transcription and replication machineries, MTERF might provide the replication machinery with a 3´ end that is capable of priming lagging-strand DNA synthesis and simultaneously providing a new RNA bootlace so that the replication fork can continue replication in the forward direction.

6.5.2 A possible role for MTERF in preventing the head on collision of the transcription and replication machineries

The sea urchin MTERF homologue mtDBP exhibits bidirectional contrahelicase activity, suggesting that it might act as a negative regulator of replication fork progression. This

raises the question of whether, human MTERF might have an analogous function in mtDNA metabolism in addition to any possible role in transcription termination (Loguercio Polosa *et al*. 2005). mtDBP regulates transcription termination *in vitro* only when the enzyme approaches the DNA bound mtDBP from the L-strand direction and therefore mtDBP is suggested to act as a polar termination factor.

I therefore propose that MTERF promotes replication pausing on a template molecule while transcription is occurring at the same time. MTERF could therefore be acting as a "traffic signal" regulating the orderly passage of the replication and transcription machineries. Such regulation maybe important since unregulated collisions can create recombinogenic ends and lead to genomic instability. Such a regulation mechanism has been reported at least in *Escerichia coli* (Neylon *et al*. 2005, Mulcair *et al*. 2006). Loguercio Polosa *et al*. (2005) suggested that in sea urchin the passage of the RNA polymerase through the DNA-mtDBP complex abrogates the mtDBP contrahelicase activity.

The *Escerichia coli* Tus protein provides an excellent model for studying the human MTERF protein despite the lack of structural homology. *E. coli* has several replication fork arrest sites (Hill *et al*. 1988) which act in a polar manner, as in prokaryotes generally (Hill *et al*. 1989). Replication forks are arrested by the terminator protein, Tus, binding to its target site (Hill *et al*. 1989, Neylon *et al*. 2005). Tus shows contrahelicase activity only in one direction and has also been shown to act as a polar block for elongating transcripts *in vitro* (Mulcair *et al*. 2006). The physiological significance of this polar RNA chain blocking activity lies in its ability to abrogate contrahelicase activity, as observed *in vitro* (Mohanty *et al*. 1996). *In vivo* migration of a transcription complex through the ter sequence also abrogates the contrahelicase activity, and replication forks are subsequently enabled to progress. On the other hand replication fork arrest is not abrogated when transcription approaches the ter site towards the blocking end (Mohanty *et al*. 1996). Similarly to *Escerichia coli* Tus protein, MTERF binds on both sides of the replication terminus region and is here proposed to regulate the passage of transcription/replication machineries approaching the terminus region from opposite

directions, thus preventing them colliding with each other head-on. Unregulated head-on collision of two protein machineries can lead to genomic instability in prokaryotic genomes (Takeuchi *et al*. 2003, Prado and Aguilera 2005) and prevents DNA replication whereas in the nucleus of mammalian cells it leads to formation of HSRs (homogeneously staining regions of chromosomes) (Hashizume and Shimizu 2007).

DmTTF has two binding sites in *Drosophila* mtDNA and transcription termination has been shown to take place at these sites. *In vitro* studies carried out on DmTTF have shown that it is capable of terminating transcription. However, silencing DmTTF has shown that it has no role in generating the 3´ ends of the mature transcripts. Roberti *et al*. (2005) reported that *in vitro* DmTTF has bidirectional transcription termination activity with biased polarity. DmTTF protein was therefore found to act asymmetrically so that it exposes different faces to RNA polymerases that approach it from different directions (Roberti *et al*. 2005). Thus it is possible that DmTTF could have a role in mtDNA replication as well, as hypothesized for mtDBP (Loguercio Polosa *et al*. 2005) and here for MTERF.

6.6 Does MTERF have a role in MELAS pathogenesis?

The functional role of MTERF is interesting because the A3243G MELAS mutation is situated in the middle of its canonical binding site within the $tRNA^{Leu(UUR)}$ gene. The mutation has been shown, *in vitro*, to dramatically reduce the binding of MTERF to its target site and to suppress transcription termination efficiency (Hess *et al*. 1991, Chomyn *et al*. 1992). However, it remains to be established whether MTERF finally has a role in MELAS pathogenesis. Some MELAS-causing mutations in the $tRNA^{Leu(UUR)}$ gene are not located within the MTERF binding sequence and some of them are even located in other genes where no alteration in MTERF binding is seen. Nevertheless, it is possible that when the A3243G MELAS mutation is considered, interaction with MTERF might still be necessary. I showed that altering MTERF expression levels affects antisense transcript levels; it is possible that these antisense transcripts might play a role in MELAS

pathogenesis. It should be interesting to study whether antisense transcript levels in A3242G mutation carrying cells differ from those in normal cells.

The A3242G MELAS mutation and silencing of MTERF by RNAi lead to the same end result, namely the MTERF binding to its target sequence is potentially decreased. My results indicate that MTERF does influence mitochondrial transcription *in vivo* and thus it cannot be ruled out that some of the pathological effects seen in MELAS may be due to impaired transcription termination or modified relative amounts of sense- and antisense-strand transcripts.

6.7 The physiological role of MTERF and its crystal structure

The MTERF structure has been solved only recently and it has further elucidated the possible biological significance of the MTERF protein and also other members of the MTERF protein family since they also have the MTERF motif. The MTERF structure consists of nine left-handed helical MTERF motifs that form a solenoid structure which is further twisted to the right. MTERF apparently has two DNA binding sites and it binds one continuous DNA strand creating a bend in DNA (Jiménez-Menéndez *et al*. 2010).

DNA bending has been shown to be important for transcription termination. One other transcription termination factor, TTF1, has been reported to induce DNA bending of 40° (Smid *et al*. 1992) and similarly to bring about transcription termination (Kuhn *et al*. 1990). TTF1 has also been reported to show contrahelicase activity (Pütter and Grummt 2002), like the sea urchin MTERF homologue mtDBP (Loguercio Polosa *et al*. 2005). We have shown that over-expressing MTERF promotes mtDNA replication pausing and therefore implicating it in replication as well. The MTERF molecular structure and its ability to bend DNA are consistent with the idea that it might indeed have a dual role in mtDNA transcription and replication.

Jiménez-Menéndez (2010) established that repeats I-VI of the full length MTERF protein bind one half of the binding site oligonucleotide and that repeats VIII and IX bind the other half of it. They showed that two conserved guanines contact two arginines at positions 169 and 202. These guanine residues are G12 from strand C and G3 from strand B which I established to be part of the minimal binding site of MTERF by means of SELEX. In addition G4 from strand B contacts Glu165. It appears that full length MTERF protein binds YGGY at both ends of its minimal binding site, thus creating a bend in the mtDNA.

Since full length MTERF protein is able to bind DNA at two sites it is conceivable that it can create a loop in mtDNA and might therefore have a role in regulating the relative activities of the PH1 and PH2 transcription units and explain the difference in rRNA and mRNA levels in mitochondria after all as proposed by Martin *et al*. (2005).

Jiménez-Menéndez *et al*. (2010) also reported that MTERF protein has a shorter variant that is due to spontaneous proteolysis of the full length MTERF protein. This shorter version of MTERF, MTERF-ΔN, is also capable of binding DNA but does so in a sequence non-specific manner contacting the DNA in the major groove. It is possible that MTERF-ΔN somehow interacts with the full-length protein that binds DNA in a sequence specific manner.

6.8 MTERFD1 and MTERFD3 binding to human mtDNA

The third aim of my study was to investigate two recently identified members of the human MTERF protein family. MTERFD1 and MTERFD3 were found to be efficiently expressed in the inducible Flp-In™ T-Rex™-293 cells and were mitochondrially targeted as predicted. I was not able to establish any sequence-specific DNA binding activity for either of the proteins, which is consistent with other published data which showed little or no sequence-specific binding for either factor *in vitro* or *in vivo* (Park *et al*. 2007, Pellegrini *et al*. 2009, Wenz *et al*. 2009). Nevertheless, I detected that the over-expressed

MTERFD3-HA complex was supershifted with the anti-HA antibody when probed with four separate probes located at the O_H/promoter region, with which TFAM has also been shown to interact. It is therefore possible that MTERFD3 interacts with the TFAM-DNA complex. Over-expressing MTERFD3-HA did not enhance the signal. However, MTERFD3 is already an abundant protein and this might explain the absence of any visible effect on the signal seen in EMSA.

6.9 Physiological role of MTERFD1 and MTERFD3 in human mitochondrial DNA maintenance

I investigated the effect of modulating MTERFD1 and MTERFD3 protein levels on mtDNA copynumber. Over-expression of either of the proteins leads to mtDNA copynumber depletion, suggesting that they might have a role in mtDNA replication. I continued by studying the effect of over-expressing the epitope-tagged proteins on mitochondrial DNA replication intermediates by means of 2DNAGE. The effect of over-expressing either of the proteins was distinct from the one due to MTERF over-expression; complex junctional molecules were upregulated whereas the amount of gapped circles was downregulated when the DNA was analysed by *Pvu*II digestion. Conversely, when either of the two proteins was silenced by means of RNAi, incomplete circles were increased compared with other replication intermediates.

There are also earlier studies suggesting that MTERFD1 might play a role in mtDNA replication since *Mterfd1*-mice do not survive past embryonic stage (Park *et al*. 2007). This is also supported by my mtDNA copynumber analysis data. On the other hand, knocking out *Mterfd3* in the mouse did not result in a dramatic outcome but a mild, rather late-onset pathology (Wenz *et al*. 2009) which is again consistent with my mtDNA copynumber data. Given the similar effects on replication intermediates, I suggest that these two proteins might co-operate in some way. One possible molecular mechanism for that would be that MTERFD3 is actually an accessory protein for MTERFD1.

Above I introduced the idea that MTERF, like mtDBP, might have contrahelicase activity. My findings on the biological role of MTERFD3 suggest that it could also regulate replication fork passage, but immediately upstream of the replication terminus, since it appears to interact preferentially with the NCR at the O_H/promoter region, causing an accumulation of pre-termination mtRIs. As over-expressing MTERFD1 leads to similar but less dramatic changes in the mtRIs, I suggest that it could cause a similar kind of effect but more widely in the genome. RNAi of either MTERFD1 or MTERFD3 yielded results that are in accordance with the possibility that these proteins show contrahelicase activity; the above mentioned gapped circles being exactly what we would expect to see if replication fork impedance in the pre-termination zone is alleviated.

6.10 TFAM and MTERF have distinct effects on mitochondrial transcription and replication intermediates

We studied the effect of modulating TFAM levels on mitochondrial transcription and replication. We established that both over-expression and silencing TFAM decrease mtDNA copynumber whereas modulating MTERF levels did not affect mtDNA copynumber and that over-expression of TFAM down regulates mitochondrial transcripts. When over-expressed, TFAM led to an increase in the amount of RIs in general, most clearly in the rDNA region, and it also caused a decrease in replication termination intermediates. When TFAM was silenced it led to enhancing of termination intermediates but RIs did not shift from the ribosubstituted type more generally. Treating cells with dideoxycytidine led to a generally similar phenotype as TFAM over-expresssion at large.

Many conflicting findings have been published on the proposed role of TFAM in packaging the mtDNA (Takamatsu *et al*. 2002, Alam *et al*. 2003, Ekstrand *et al*. 2004, Kanki *et al*. 2004a, Kanki *et al*. 2004b, Wiesner *et al*. 2006, Cotney *et al*. 2007). One theory is that the mtDNA is packed with TFAM and saturated with it (Takamatsu *et al*. 2002, Alam *et al*. 2003, Kanki *et al*. 2004a, Kanki *et al*. 2004b). Takamatsu *et al*. (2002)

reported that the human TFAM:mtDNA ratio is 1700:1 which they find to be of the same order of magnitude as in *Xenopus laevis* oocytes (2000:1). Cotney *et al*. (2007) reported a TFAM:mtDNA ratio of 50±8:1, i.e. of the same order of magnitude as the 35:1 reported by Wiesner *et al*. (2006). There are also many contradictory findings about the effect of manipulating TFAM levels on mitochondrial transcription. Originally TFAM was characterized to be a mitochondrial transcription factor (Fisher and Clayton 1988). Upregulating TFAM has been reported to increase *in vitro* the levels of run-off transcripts from mtDNA promoters (Parisi and Clayton 1991, Falkenberg *et al*. 2002) but, but now it seems that when expressed at very high levels it can also suppress transcription (Falkenberg *et al*. 2002, Maniura-Weber *et al*. 2004). Our findings on the effect of over-expressing TFAM in transcription are supported by those of Falkenberg *et al*. (2002) and Maniura-Weber *et al*. (2004). However, if the mtDNA was already packed with TFAM our findings could possibly be difficult to explain. Namely, if the mtDNA was already heavily saturated with TFAM, the overexpression of TFAM should not have any effect on transcript levels. However, it is possible that a simple titration model cannot be directly applied to explain the mechanism by which TFAM regulates mtDNA copynumber. It has been observed that, during the recovery period after ethidium bromide-induced mtDNA depletion in cultured cells, the mtDNA levels recovered faster than TFAM levels, suggesting that the packing ratio of TFAM on mtDNA may actually vary and also affect mtDNA replication (Seidel-Rogol and Shadel 2002). Finally, Shutt *et al*. (2010) showed that specific transcription initiation can occur *in vitro* from both LSP and HSP1 independently of TFAM. Clearly, further studies need to be carried out to elucidate the role of TFAM in mitochondrial transcription.

Both TFAM and MTERF seem to have different roles in mitochondrial transcription, opposing the ideas previously presented. Similarly, the role of MTERF in transcription termination should be reconsidered, as modulating MTERF levels had a clear effect only on antisense transcript levels and the effects on mature mitochondrial transcripts *in vivo* appear to be negated or modified by compensatory mechanisms.

TFAM does not show sequence specificity when binding mtDNA but binds preferentially to branched DNA structures (Ohno *et al*. 2000) and to cisplatin-damaged or oxidized DNA (Chow *et al*. 1994, Chow *et al*. 1995, Yoshida *et al*. 2002). However, TFAM also shows preferential binding to the region upstream of the LSP, giving a footprint (Fisher and Clayton 1988, Ghivizzani *et al*. 1994), but both mature human and mouse TFAM proteins also bind dsDNA nonspecifically with high affinity (Ekstrand *et al*. 2004). Even if TFAM does not bind DNA in a sequence-specific manner, it is possible that it could enhance replication pausing by causing a bend in DNA like *Schizosaccharomyces pombe* Sap1p has been suggested to do (Krings and Bastia 2005). The pause sites TFAM over-expression enhances are rather diffuse compared to pause sites enhanced by MTERF over-expression. Generally over-expressing MTERF has more site specific effects on mitochondrial replication than TFAM as it binds mtDNA in a sequence specific manner in contrast to TFAM. MTERF and TFAM enhance replication pausing at some of the same sites; however, it remains to be investigated whether, and if so how, they interact in regulating transcription and replication pausing.

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The mitochondrial transcription termination factor mTERF modulates replication pausing in human mitochondrial DNA

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ABSTRACT

The mammalian mitochondrial transcription termination factor mTERF binds with high affinity to a site within the tRNA$^{Leu(UUR)}$ gene and regulates the amount of read through transcription from the ribosomal DNA into the remaining genes of the major coding strand of mitochondrial DNA (mtDNA). Electrophoretic mobility shift assays (EMSA) and SELEX, using mitochondrial protein extracts from cells induced to overexpress mTERF, revealed novel, weaker mTERF-binding sites, clustered in several regions of mtDNA, notably in the major non-coding region (NCR). Such binding *in vivo* was supported by mtDNA immunoprecipitation. Two-dimensional neutral agarose gel electrophoresis (2DNAGE) and 5′ end mapping by ligation-mediated PCR (LM-PCR) identified the region of the canonical mTERF-binding site as a replication pause site. The strength of pausing was modulated by the expression level of mTERF. mTERF overexpression also affected replication pausing in other regions of the genome in which mTERF binding was found. These results indicate a role for TERF in mtDNA replication, in addition to its role in transcription. We suggest that mTERF could provide a system for coordinating the passage of replication and transcription complexes, analogous with replication pause-region binding proteins in other systems, whose main role is to safeguard the integrity of the genome whilst facilitating its efficient expression.

INTRODUCTION

The mitochondrial genome of animals is organized in a highly compact manner, with virtually no non-coding information between or within its 37 genes. The circular genome is transcribed by a phage-type RNA polymerase into polycistronic transcripts which, in mammals, encompass the entire genome on both strands (1,2). Production of these transcripts depends upon a set of closely spaced promoters located in the major non-coding region (NCR). The primary transcripts are then processed to mature mRNAs, rRNAs and tRNAs via a series of enzymatic steps requiring the tRNA-processing endonucleases RNase P and tRNAse Z, as well as other enzymes. The major coding strand (informationally the L-strand, but for the purposes of transcription conventionally referred to by the name of the template, H-strand) is transcribed from two distinct initiation sites at the heavy-strand promoter (HSP), P_{H1} and P_{H2}, separated by ~100 bp. The P_{H2}-derived precursor transcript covers virtually the entire genome and can give rise to all of the transcription products of the heavy-strand except tRNA^{Phe}, whose coding sequence overlaps the P_{H2} initiation site. The P_{H1} initiation site gives rise to a truncated transcript encompassing just the rRNAs (plus two tRNAs) and thus defines a distinct mitochondrial rDNA transcription unit.

Termination at the 3′ end of the rDNA is brought about by a transcription termination factor, mTERF (3–6), which has also been proposed to interact with the RNA polymerase in initiation site selection (2,7). Recent data suggest that this involves formation of a DNA loop in which RNA polymerase complexes are recycled around the rDNA segment of the genome after terminating (7). mTERF binds sequence specifically with high affinity to a

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sequence element within the coding sequence of $tRNA^{Leu(UUR)}$, located immediately downstream of the rDNA (4). Current evidence indicates that mTERF interacts with its asymmetric-binding site as a monomer (8), although the tertiary structure of the protein and the structural basis of its interaction with DNA are unknown.

mTERF belongs to a recently identified superfamily of proteins whose functions are largely unknown (9–11). Homologues in *Drosophila* and in sea urchins have variously been implicated in transcriptional termination (12–14), regulation of DNA replication (15) and even mitochondrial protein synthesis (11). The sea urchin mTERF homologue mtDBP (D-loop-binding protein) has recently been shown to terminate transcription in a polar manner (14,16), analogous with the activity of mTERF (5). However, mtDBP is also a contrahelicase (15), and has been proposed to play a role in regulating the expansion of the short D-loop of sea urchin mtDNA and thus the initiation of productive replication of the genome.

Transcription and replication of mtDNA have long been regarded as interlinked processes. The primer for initiation of DNA replication has been assumed to be a product of transcription by the mitochondrial RNA polymerase. However, there is no consensus concerning the mechanism by which 3′ ends are generated for extension by DNA polymerase, variously proposed to be RNA processing by endonuclease MRP (17) or protein-independent termination at one of the conserved sequence blocks of the NCR (18). The exact site of replication initiation is also unclear, and may vary between cell-types. A prominent cluster of 5′ ends in H-strand DNA, designated as O_H, is generally regarded as the major origin of (unidirectional) replication. However, there is no direct experimental evidence that it functions thus, and bidirectional initiation clearly occurs in some molecules at sites downstream of O_H, both in cultured cells (19), especially when recovering from drug-induced mtDNA depletion (20), and in solid tissues (21,22). In a minority of molecules (21) these initiation sites can encompass the entire genome (in birds) or almost the entire genome (in mammals).

Mitochondrial DNA (mtDNA) was for over 25 years assumed to replicate by a unique, strand-asynchronous mechanism (23). However, more recent analysis of mtDNA replication intermediates (RIs) by two-dimensional neutral agarose gel electrophoresis (2DNAGE) failed to detect the extensively single-stranded products of such a replication mechanism (19–22,24–26) and instead revealed two classes of double-stranded RIs. One class consists of the predicted products of conventional strand-coupled replication (19,21,24,25); the other contains extended RNA segments (26) encompassing the entire lagging strand (RITOLS, 'RNA incorporation throughout the lagging strand', 19). Maturation of the lagging strand to DNA appears to occur with different kinetics and distinct sites of initiation in different organisms (19), and some RIs of the first class could be interpreted as molecules in which lagging-strand DNA synthesis has effectively caught up with the advancing fork, as a result of replication pausing. The mechanism by which the RNA lagging strand is created is unknown.

mtDNA replication also depends on the HMG-box protein TFAM, named for its essential role as a cofactor for efficient and specific transcriptional initiation. TFAM is required for mtDNA maintenance (27) and appears to have several distinct roles in mtDNA metabolism. It is a major structural protein of the mitochondrial chromosome, but also influences mtDNA replication in ways connected with transcription. Overexpression of TFAM leads to a drop in mitochondrial transcript levels and a pronounced shift toward conventional, strand-coupled RIs (28). This could represent either a general slowing or stuttering of fork advance, attributable to a decreased availability of RNA to form the lagging strand (i.e. in which lagging-strand maturation frequently catches up with fork advance) or else a programmed switch to standard DNA synthesis.

As a factor affecting the outcome of mitochondrial transcription, mTERF might be expected also to have some influence over mtDNA replication if, as suggested, replication is intimately connected with transcription. We therefore embarked on a series of experiments to document the effects on mtDNA replication of modulating the expression of mTERF in cultured human cells. To this end, we set out initially to characterize better the binding specificity of mTERF, especially given recent reports of possible additional binding sites for mTERF *in vivo* (6,29). Electrophoretic mobility shift assays (EMSA) using mitochondrial protein extracts from cells induced to overexpress mTERF revealed additional, though weaker mTERF-binding sites clustered in strategically important regions of the mitochondrial genome. 2DNAGE and lagging strand 5′ end mapping by ligation-mediated PCR (LM-PCR) identified the canonical mTERF-binding site as a replication pause site, with the frequency of pausing subject to the expression level of mTERF. Replication pausing in other regions of the genome, notably the NCR, was also affected by mTERF overexpression. These results indicate a role for mTERF in mtDNA replication, in addition to its role in transcription.

MATERIALS AND METHODS

Cells and cell culture

Human embryonic kidney-derived HEK293T cells, Flp-In™ T-Rex™-293 cells (Invitrogen), 143B osteosarcoma, Jurkat and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) containing 4.5 g/l of D-glucose, 10% foetal calf serum (Sigma), 50 μg/ml uridine (Sigma) and 2 mM L-glutamine (BioWhittaker/Cambrex) at 37°C in an incubator with 5% CO_2 in air. Flp-In™ T-Rex™-293-derived cell-lines were cultured under selection with blasticidin and hygromycin according to the manufacturer's protocol. Expression of mTERF or mTERF-MycHis (C-terminally tagged) was induced in transfected Flp-In™ T-Rex™-293 cells with 10 ng/ml doxycyclin (Sigma-Aldrich), which was replenished every 48 h. Cells were passaged routinely

every 3–4 days at 1:10 or 1:20 dilution. Adherent cells were detached either by pipetting alone or, for HeLa cells, by treatment with Trypsin-EDTA (Bio-Whittaker/Cambrex). Suspension cells (Jurkat) were passaged by centrifugation and resuspension in fresh medium.

Oligonucleotides and plasmids

Oligonucleotides used to create EMSA or hybridization probes by PCR from purified human mtDNA or cloned segments thereof are listed in Supplementary Table 1. The mTERF coding region, including the mitochondrial targeting signal and 24 nt of the 5′ untranslated region, a total of 1221 bp, was amplified from HeLa cell cDNA (30) using the following chimeric primer pairs (all sequences shown 5′ to 3′, restriction sites used for cloning underlined): BamHI-mTERF F1, CGCGGATCCCTGTTCTC CAGCCTTTCTGG plus HindIII-mTERF R1, CCC AAGCTTGGCAAATCTGCTTAACTTTTT to create an in-frame C-terminal fusion to the Myc epitope tag; BamHI-mTERF F1 plus HindIII-mTERF R STOP, CCCAAGCTT*TCA*GGCAAATCTGCTTAACTTTTTC to create an mTERF expression construct containing the stop codon at the natural position (shown in italics, underlined, complementary strand). After sequence verification PCR products were digested with BamHI and HindIII (Fermentas, manufacturer's recommended conditions) and ligated to similarly digested pcDNA3.1(-)/Myc-His A (Invitrogen) vector DNA to create the mTERF and mTERF-MycHis expression constructs. For induced expression using the Flp-In™ T-REx™-293 cell system these plasmids were digested with PmeI (New England Biolabs), which cuts on either side of the insert, ligated into PmeI-digested DNA of the vector pcDNA5/FRT/TO (Invitrogen), and stably transfected into the recipient cells as previously (31).

DNA and siRNA transfections

HEK293T cells were transfected either with 3 μg of plasmid DNA and 30 μl of LipofectAMINE (Invitrogen) or 10 μg of DNA and 40 μl of TransFectin™ Lipid Reagent (Bio-Rad), according to manufacturers' protocols. Transiently transfected cells were subsequently harvested for different assays, or placed under selection using 2 mg/ml G418 Sulfate (Calbiochem), in order to select clones of stably transfected cells expressing mTERF-MycHis. mTERF-specific siRNAs were synthesized by means of *in vitro* transcription using the Silencer™ siRNA construction kit (Ambion). Candidate target sites for specific mTERF silencing were chosen using a prediction programme provided by Ambion (http://www.ambion.com/techlib/misc/siRNA_finder.html). One out of five tested siRNAs was found to be efficient in mTERF silencing (see Results section), the relevant target site in mTERF mRNA being nt 585–605 (5′-AAGCGGGUGAAAGCUAACAUU-3′). To knockdown mTERF expression, HEK293T cells (with or without prior stable transfection with the mTERF-MycHis expression construct) were transfected with 10 nM (final concentration) of mTERF-specific siRNA molecules using Lipofectamine™ 2000 transfection reagent (Invitrogen), as per manufacturer's recommendations. An siRNA reagent targeted on 5′-GGAGAAGGUACGAGGGGC AUU-3′ (siRNA Control) was used as a negative control.

Immunocytochemistry

For immunocytochemistry cells were grown on coverslips, seeded at low density. Twenty-four hours after transfection or induction with doxycyclin, cells were washed with DMEM and then incubated in fresh medium containing 100 nM MitoTracker® Red CMXRos (Molecular Probes) at 37°C for 10 min, then washed twice with PBS. After incubation in fresh medium at 37°C for 2 h, cells were again washed twice with PBS and fixed in 4% formaldehyde/5% sucrose in PBS at 37°C for 15 min. After three further PBS washes cells were permeabilized by incubation in 0.5% Triton X-100 in PBS at 37°C for 15 min, washed twice with PBS, incubated in blocking solution (5% w/v non-fat milk powder in PBS) at room temperature for 45 min, then again washed three times with PBS. After incubation in primary antibody solution, mouse anti-Myc monoclonal 9E10 (Roche Molecular Biochemicals, stock 5 mg/ml) 1:1000 in PBS for 1 h at room temperature, cells were washed three times with PBS, then incubated for 1 h at room temperature in a 1:200 dilution of secondary antibody, fluorescein-conjugated horse anti-mouse IgG (Vector Technologies, stock 1.5 mg/ml). After three final PBS washes the coverslips were mounted on slides using Vectashield with DAPI (Vector Technologies). Cells were visualized and photographed using an Olympus IX70 inverted confocal microscope at 100× magnification, with excitation at 568 nm (emission 607/45) for Mitotracker Red and 488 nm (emission 525/50) for fluorescein, using an Andor iXon DV885 front-illuminated CCD camera.

Western blotting

SDS–PAGE used 12% polyacrylamide gels under standard conditions (32). Protein extraction and western blotting were carried out essentially as described previously (30). Primary antibodies used were mouse anti-Myc monoclonal 9E10 (as above, diluted 1:15000) and rabbit anti-human mTERF antibody, custom-supplied (Invitrogen) as an anti-peptide (KLH-conjugated CSNDYARRSYANIKE) antibody, 1 mg/ml, diluted 1:5000. Kodak Biomax™ ML X-ray film was exposed to the filter membrane for between 5 s and 5 min.

Preparation of mitochondrial lysates

Cells were harvested without trypsinization, resuspended in 1 ml (per 10 cm plate of cells) of resuspension buffer (0.133 M NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 25 mM Tris–HCl pH 7.5) and centrifuged at $1200g_{max}$ for 2 min at 4°C. The pellet was resuspended in 0.5 ml of swelling solution (10 mM NaCl, 1.5 mM $CaCl_2$, 10 mM Tris–HCl pH 7.5) and incubated on ice for 15 min. After swelling, the cells were dounce-homogenized (20–25 strokes, tight-fitting pestle) on ice and breakage of the cells was checked microscopically. An equal volume of sterile filtered sucrose/EDTA buffer (0.68 M sucrose, 2 mM EDTA, 20 mM Tris–HCl pH 7.5) was added immediately after

breaking the cells. Nuclei and debris were pelleted by centrifugation at $1200g_{max}$ for 10 min at 4°C. The supernatant was transferred to a fresh tube and centrifugation was repeated. The supernatant was collected and recentrifuged at $16\,000g_{max}$ for 30 min at 4°C. The mitochondrial pellet was washed once with 200 µl of PBS and frozen at −80°C or lysed immediately. For processing large quantities of cells the volumes were scaled up. Mitochondrial lysates were prepared essentially as described by Fernandez-Silva *et al.* (33), except using 'Complete, Mini protease inhibitor cocktail' (Roche) instead of PMSF.

EMSA

DNA fragments for EMSA were PCR amplified using mtDNA as template and primer pairs shown in Supplementary Table 1, followed by sequence verification of the product. dsDNA oligonucleotide probes for EMSA (Supplementary Table 1) were prepared by mixing equal amounts of complementary oligonucleotide pairs in 500 µl of H_2O to a final concentration of 2 mM, followed by incubation for 5 min at 100°C and cooling to room temperature on the bench. Total of 300 ng of each PCR fragment or 20 pmol of each dsDNA oligonucleotide were labelled using 8 U of T4 polynucleotide kinase (Fermentas) and 15 µCi of [γ-^{32}P] ATP (Amersham Pharmacia Biotech, 3000 Ci/mmol) in 15 µl final volume of PNK buffer (MBI Fermentas). Reactions were stopped on ice and diluted to 100 µl with H_2O. EMSA was carried out in 20 µl binding reactions according to Fernandez-Silva *et al.* (33) with minor modifications. Reactions contained at least 10 µl of the binding buffer (25 mM HEPES–KOH, pH 7.5, 12.5 mM $MgCl_2$, 20% glycerol, 0.1% Tween-20, 1 mM DTT), 0.2 pmol of labelled dsDNA oligonucleotide or 3 ng of labelled PCR product as probe, 5 µg of mitochondrial lysate, 100 mM KCl, 5 µg BSA and 5 µg of non-specific competitor DNA poly(dI-dC)–(dI-dC) (Amersham Pharmacia Biotech). Reactions were incubated at room temperature for 20 min and terminated on ice with addition of 0.25 volumes of 30% glycerol. Competition EMSA reactions contained also up to 100-fold excess of the non-labelled competing probe. Supershift EMSA reactions contained 0.5 µg of anti-Myc antibody (as above), or 1 µg of anti-FLAG® M2 antibody (Sigma), which was added 30 min prior to the labelled probe. Depending on the length of the fragment, reaction products were analysed on 5–10% non-denaturing polyacrylamide TBE gels, pre-run at 4°C in 2.2× TBE at 100 V for 1 h at 4°C, then run at 100 V for 30 min and 175 V for 3–5 h depending on the size of the probe fragment. Gels were dried and autoradiographed using KODAK BioMax™ MS film.

SELEX

Creation of a randomized DNA ligand library was carried out essentially as described by Blackwell (34). The 46 nt long oligonucleotide template contained 14 internal random nucleotides, flanked on either side by 16 nt fixed ends corresponding with standard primers, containing recognition sites for BamHI and EcoRI, respectively. Second-strand synthesis was carried out in a reaction volume of 20 µl containing 1.6 µg of template, 500 µmol of primer, 2 mM dNTPs and 5 U of Klenow fragment (Fermentas) in Klenow fragment buffer at 46°C for 1 min, followed by 37°C for 7.5 min. The reaction was stopped by heating at 75°C for 10 min and the dsDNA ligand library was gel-purified from an EtBr-stained 14% native polyacrylamide gel using the QIAEX kit (QIAGEN) according to the manufacturer's protocol. Ligand selection was carried out in 25 µl reactions under essentially the same conditions as EMSA, using 10 µg of mitochondrial protein lysate from mTERF-MycHis expressing Flp-In™ T-Rex™-293-cells, 0.8 µg of the ligand DNA and 6.25 µg of non-specific competitor DNA poly(dI-dC)–(dI-dC) incubated for 20 min at room temperature. Pre-swollen anti-myc-Sepharose beads (Amersham Biosciences) were suspended in EMSA buffer (25 mM HEPES–KOH, pH 7.5, 12.5 mM $MgCl_2$, 20% glycerol, 0.1% Tween-20, 1 mM DTT, 100 mM KCl, 0.2 µg/µl BSA), washed once in the same buffer and resuspended in 1.5 volumes of the same buffer containing 0.25 µg/µl poly(dI-dC)–(dI-dC). To each binding reaction was added 100 µl of the bead suspension, followed by gentle rotation for 2 h at 4°C. Beads were then washed in EMSA buffer containing 0.25 µg/µl poly(dI-dC)–(dI-dC), followed by a further seven times in the buffer without poly(dI-dC)–(dI-dC) and gentle rotation overnight in 100 µl of K buffer (10 mM Tris–HCl, 0.5 mM EDTA 50 mM NaCl, pH 8.0) containing 100 µg/ml of freshly dissolved proteinase K (Fermentas). DNA was recovered from the beads by extraction with phenol–chloroform–isoamyl alcohol (25:24:1) and ethanol precipitation, washed once with 70% ethanol and resuspended in a minimal volume of H_2O (~7.5 µl). PCR was then carried out using 2 µl of this template in a 50 µl reaction volume containing 0.2 µM of each SELEX primer (GGTGAATTCGCTCACG and GAACGGATCCCTTTCG, both shown 5′ to 3′, with restriction sites for cloning underlined) and 2.5 U of Pfu DNA polymerase (Promega). Thirty amplification cycles were carried out using a 15 s extension step, after which the enriched ligand DNA was gel-purified from an EtBr-stained 12% native polyacrylamide gel as above. After seven such enrichment cycles, the ligand DNA was cloned into pCR®4Blunt-TOPO® vector (Invitrogen) and individual clones were sequenced using standard primers on an ABI 3100 sequencer using the BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems).

DNA extraction and mtDNA copy number estimation

For the preparation of mtDNA (mitochondrial nucleic acids) for analysis of RIs from cultured cells, mitochondria were isolated and processed as described by Pohjoismäki *et al.* (28). Total DNA for analysis of mtDNA copy number was extracted from cells by standard methods (35), and copy number was determined using quantitative PCR, as described previously (28), with amyloid precursor protein (APP) as a single-copy nuclear DNA standard. Human placental mtDNA was prepared as previously (24). Total DNA for analysis of mtDNA RIs

was extracted from frozen human tissue blocks ~7 × 7 × 7 mm^3) obtained via forensic autopsies. The samples were taken as part of the Tampere Coronary Study, approved by the Ethics Committee of Tampere University Hospital (DNO 1239/32/200/01) and the National Authority for Medicolegal Affairs. Heart, brain, skeletal muscle and kidney tissue samples were cut into thin slices with a sterile blade and suspended in 2 ml DNA extraction buffer (28). One-tenth volume of 10% SDS and 0.5 mg proteinase K were added. The crude homogenate was passed several times through a 5 ml pipette tip with a sawn-off end, to disperse the larger tissue fragments. The homogenate was incubated overnight with gentle swirling at 37°C. After incubation, 2 volumes of phenol–chloroform–isoamylalcohol (25:24:1) were added, and the mixture was shaken gently for 1 h. The mixture was then transferred to Eppendorf 15 ml Phase Lock Gel™ Heavy tubes and centrifuged at 5000g_{max} for 15 min. The aqueous phase was recovered and the extraction step repeated. DNA was precipitated by the addition of 0.2 volumes of 10 M ammonium acetate and 2 volumes of ethanol. The mixture was incubated on ice for 10 min and DNA was spooled out using a glass rod, washed once with 70% ethanol, air dried gently and resuspended in 300–700 µl TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0), depending on the pellet size. 2DNAGE analysis used 10 µg aliquots of heart and brain DNA and 20 µg aliquots of kidney and skeletal muscle DNA.

Two-dimensional neutral agarose gel electrophoresis

One microgram of total mitochondrial nucleic acids was used per analysis. Restriction digestions were performed following manufacturers' recommendations, except for BclI which was carried out at 37°C for double the usual reaction time. If subsequent treatment with S1 nuclease was used, DNA was first recovered by ethanol precipitation and resuspended in the appropriate reaction buffer, before treatment with 50 U S1 Nuclease (Promega) for 30 s. Reactions were stopped by the addition of an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1, pH 8.0) and immediately extracted. 2DNAGE was performed as described previously [(28), note different gel conditions for fragments in different size classes).

Radiolabelled probes and blot hybridization

For Southern hybridization, probes were created by *Pfu*-PCR, using cloned segments of human mtDNA as template (see Supplementary Table 1), and subsequently sequenced to confirm their identity. Probes were labelled using Rediprime™ II random prime labelling kit (Amersham) and [α-^{32}P] dCTP (Amersham; 3000 Ci/mmol).

LM-PCR

LM-PCR was carried out as described by Yasukawa *et al.* (20), using oligonucleotide primer sets as indicated in figure legends and as detailed in Supplementary Table 2.

Mitochondrial DNA immunoprecipitation

Cells were processed for mitochondrial DNA immunoprecipitation (mIP) essentially as described by Lu *et al.* (36). The mtDNA was sheared to fragments of average size 500–600 bp using a Sonics Vibra-Cell sonicator, 3 mm tip at 25% power for 3 × 20 s (1 s on, 1 s off) with incubation on ice for 30 s between. Complete, Mini protease inhibitor cocktail (Roche) was included in the lysis buffer. Lysates were pre-cleared with pre-swollen Protein A Sepharose (Amersham Biosciences) and immunoprecipitations were carried out with 5 µg mouse anti-Myc monoclonal 9E10 (Roche Molecular Biochemicals) or anti-FLAG® M2 antibody (Sigma) overnight at 4°C. Final PCR reactions used primers listed in Supplementary Table 1 and the minimum number of amplification cycles required to generate substantial product bands from the input DNAs (generally 25–26 cycles, depending on the fragment, based on preliminary tests), thus avoiding saturation.

RESULTS

mTERF has multiple binding sites in the human mitochondrial genome

In order to study the effects of mTERF on mtDNA replication, we established HEK293-derived cells expressing both natural mTERF and C-terminally Myc epitope-tagged mTERF. Mitochondrial targeting was verified by immunocytochemistry of transiently transfected HEK293T cells expressing mTERF-MycHis (Figure 1a). Induction of protein expression in Flp-In™ T-Rex™-293 cells stably transfected with the mTERF or mTERF-MycHis constructs was verified by western blotting (Figure 1b). Protein levels were the same after 24 or 48 h of induction. Prolonged overexpression of mTERF (6 days) had no significant effect on mtDNA copy number as estimated by Southern blotting (data not shown) or by quantitative PCR (Supplementary Figure 1a).

Since the main aim of the study was to determine the effects of altered mTERF expression on mtDNA replication *in vivo*, we first tested the effects of mTERF expression on protein binding to mitochondrial DNA, using EMSA with mitochondrial protein extracts from cells overexpressing mTERF. In contrast to earlier studies using purified, bacterially expressed mTERF, this tests the effects of altered mTERF expression level on protein–DNA interactions in the mitochondrial milieu, in which other mitochondrial proteins, including TFAM, are present and may influence binding.

Using EMSA with probes covering the previously identified, canonical mTERF-binding site in the $tRNA^{Leu(UUR)}$ gene, we confirmed that overexpression of natural mTERF, whether by transient or stable transfection (data not shown), or under tetracycline induction (Figure 1c), leads to a large increase in sequence-specific binding activity. The protein complex formed from the Myc epitope-tagged protein migrated slightly slower than the complex formed by endogenous

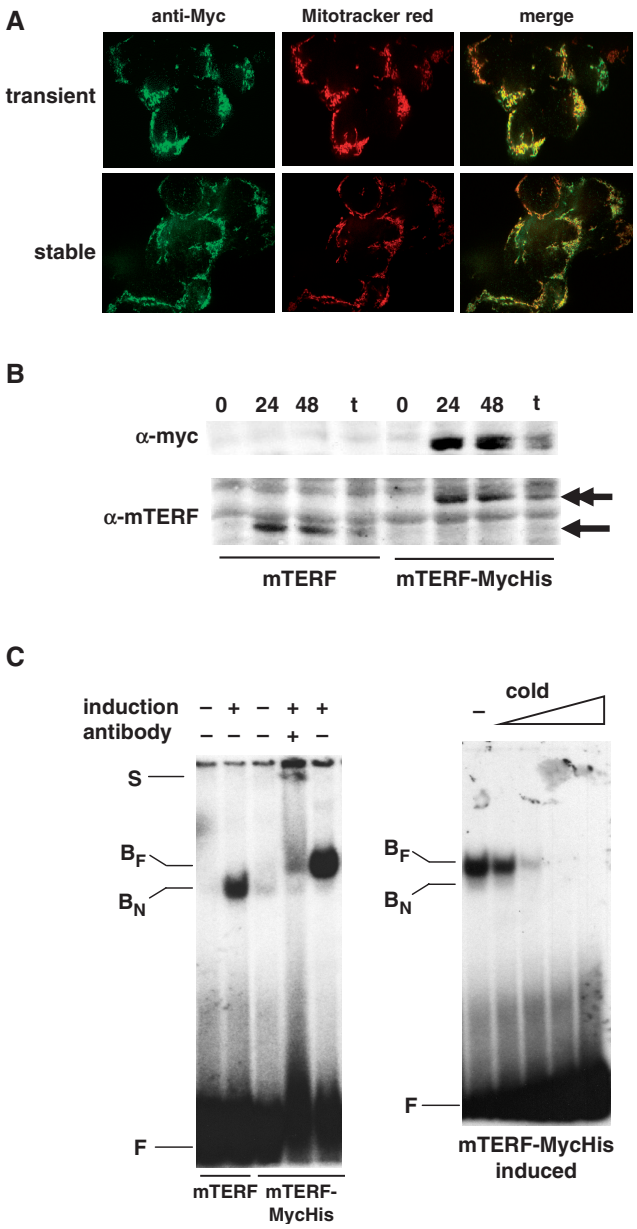

Figure 1. Overexpression of mTERF in cultured cells. (**A**) Immunocytochemistry of HEK293T cells transiently or stably transfected with mTERF-MycHis, using anti-Myc monoclonal antibody, counterstained with Mitotracker Red. (**B**) Western blots of mitochondrial protein extracts from Flp-In™ T-REx™-293 cells transfected with the mTERF or mTERF-MycHis constructs and induced for expression as indicated (0, 24, 48 h) or from transiently transfected (t) HEK293T cells, probed with anti-Myc or anti-mTERF antibodies, as indicated. The endogenous mTERF protein detected by the anti-mTERF antibody is singly arrowed. The mTERF-MycHis fusion protein detected by the same antibody is indicated by a double arrow. (**C**) EMSA using Leu-short dsDNA oligonucleotide probe and mitochondrial protein extracts from Flp-In™ T-REx™-293 cells transfected with the mTERF or mTERF-MycHis constructs and induced for expression as indicated. EMSA was carried out with or without anti-Myc antibody as shown (left-hand panel), or (right-hand panel) in the presence of an increasing amount of cold Leu-short dsDNA oligonucleotide competitor (1-, 10-, 100- and 1000-fold mass excess) or without competitor (−). The free probe (F), complexes formed by natural mTERF (B_N) or the mTERF-MycHis fusion protein (B_F), and the antibody-supershifted complex (S) are indicated. See also Supplementary Figure 1.

or overexpressed natural mTERF, and was supershifted by an anti-Myc monoclonal antibody (Figure 1c), but not by other antibodies (e.g. anti-FLAG, Supplementary Figure 1b). The anti-Myc antibody did not supershift the complex formed by endogenous or overexpressed natural mTERF (Supplementary Figure 1b).

These properties next allowed us to test other regions of the mitochondrial genome for specific binding of mTERF to DNA, using EMSA. Using overlapping fragments of ~150 bp, we scanned the major NCR and its flanking sequences, the minor NCR (O_L), its surrounding tRNA gene cluster, the region extending from O_L to the canonical mTERF-binding site in the $tRNA^{Leu(UUR)}$ gene, the ATPase 6 gene and its junction with the COIII gene and several other segments of the genome (Figure 2, Supplementary Figures 2 and 3). We estimated relative binding affinities using competition EMSA against the $tRNA^{Leu(UUR)}$ gene fragment and vice versa. As shown in Figure 2 and Supplementary Figure 2, we identified a cluster of four moderately strong mTERF-binding sites within the ND1 coding sequence and the adjacent IQM tRNA gene cluster (see Figure 2e for summary). Competition EMSA indicated that the binding to fragment ND1.1 (Figure 2b) was between one and two orders of magnitude weaker than to the canonical binding site in $tRNA^{Leu(UUR)}$. Binding to the ND1.1 fragment was tested further, using shorter, overlapping fragments (Supplementary Figure 2d). The results suggest that fragment ND1.1 contains two distinct binding sites. We also identified a binding site adjacent to O_H (fragment OH1) at least two orders of magnitude weaker than the canonical binding site, based on competition EMSA data (data not shown), as well as four other binding sites in the D-loop portion of the NCR and one at O_L, plus a possible site at the HSP (fragment OH5, see Supplementary Figure 2e).

Alignment of the sequences of these binding sites suggested a consensus which was verified by SELEX (Table 1). Most of the SELEX output clones analysed (82/109) contained at least one match to the consensus TGGT or TYGGT, and 43 clones showed an identical or almost identical (8/9) match to the extended consensus $TGGT(N_5)TYGGT$ (or its complement). Of 28 control clones analysed, subjected to the same number of amplification cycles but without antibody selection, none matched this consensus. Comparing the SELEX consensus with the canonical mTERF-binding site in the $tRNA^{Leu(UUR)}$ gene, and with the findings of an earlier application of PCR-based selection on a smaller scale using only EMSA (37), the invariant features of the binding site would appear to be two pairs of G residues on the same strand, separated by eight nucleotides (see also Supplementary Table 3).

In order to verify that mTERF is able to bind to at least some of its non-canonical binding sites *in vivo*, we carried out semi-quantitative mIP, using a minor adaptation of the method recently published by Lu *et al.* (36). For this assay we used cells inducibly expressing mTERF-MycHis, and carried out immunoprecipitation using anti-Myc antibody, as well as a control antibody (anti-FLAG) or no antibody.

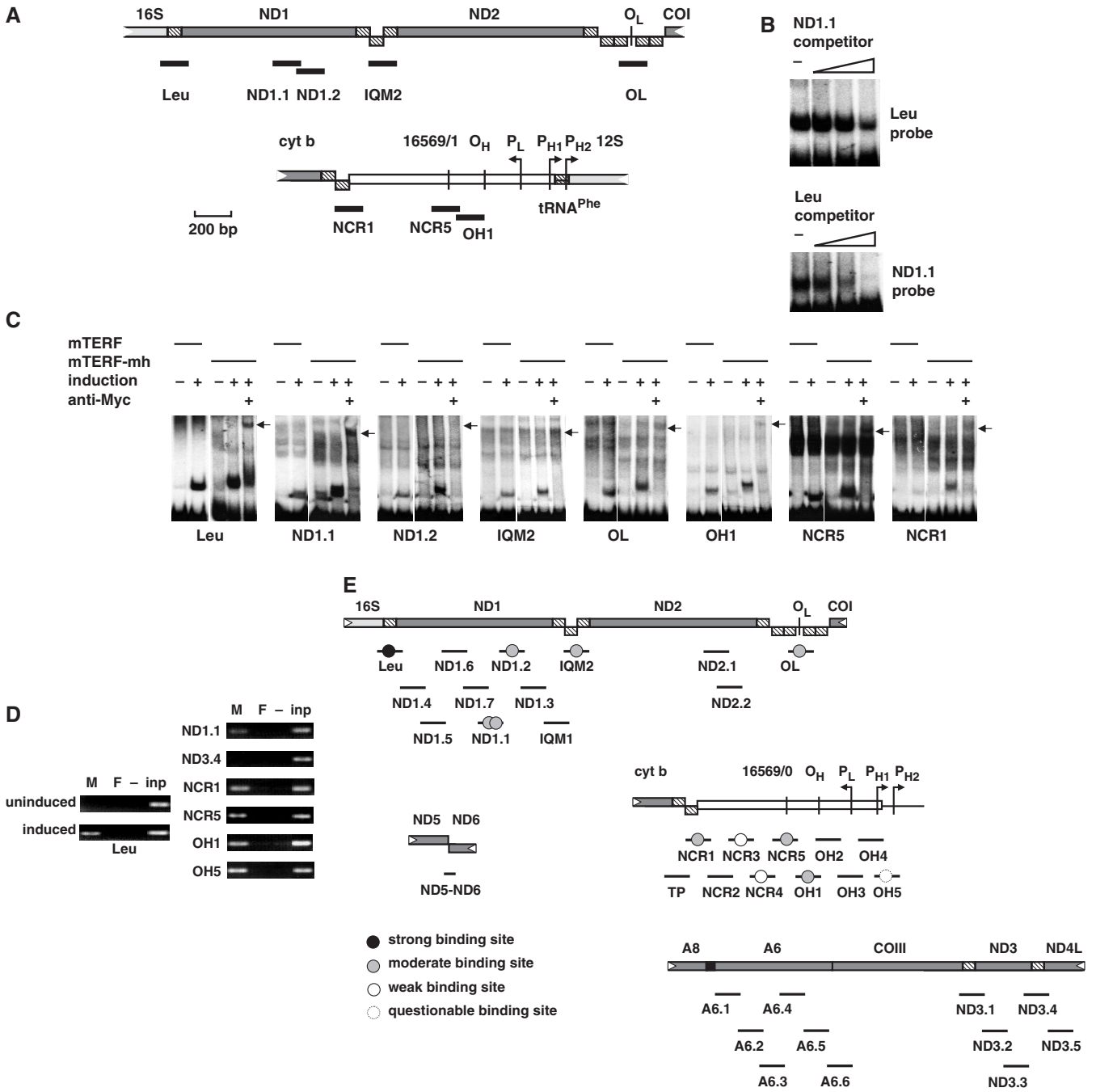

Figure 2. EMSA and mIP analysis of alternate mTERF-binding sites in human mtDNA. (**A**) Schematic diagram of regions of the mitochondrial genome in which binding was detected, showing NCR (white box), 16S and 12S rRNA genes (pale grey boxes), protein-coding genes ND1, ND2, COI and cyt b (darker grey boxes), tRNA genes (cross-hatched boxes), O_H, O_L and the promoters/transcriptional initiation sites of the two strands (P_L, P_{H1} and P_{H2}). Genes transcribed to the right shown above the centre line, genes transcribed to the left shown below. Nucleotide coordinates are as Ref. (82). Black bars indicate the positions of the 150 bp probe fragments which were found by EMSA to contain strong or moderate binding sites for mTERF, as shown in panels b and c. (**B**) Competition EMSA using the probes and competitors as shown, plus mitochondrial protein extract from cells induced to express mTERF-MycHis. The amounts of cold competitor represent 1-, 10- and 100-fold mass excess over the probe. Similar results were obtained using extracts from cells overexpressing natural mTERF (data not shown). (**C**) EMSA analyses of binding to 150 bp probe fragments as indicated, using mitochondrial protein extracts from Flp-In^{TM} T-REx^{TM}-293 cells transfected either with natural mTERF or with mTERF-MycHis (mTERF-mh) and induced for expression (or not) as indicated. Supershifting with the anti-Myc monoclonal antibody was performed for the lanes indicated. Supershifted complexes are denoted by arrows. Although the supershifted complex is minor in some cases, the main complex is always efficiently removed by the antibody, confirming the presence of mTERF-MycHis. Other antibodies tested (e.g. anti-FLAG) gave no supershifting and did not inhibit the formation of these complexes. For further experiments confirming specificity of binding and negative/weak findings using other fragments, see Supplementary Figures 1 and 2. (**D**) mIP analysis of mTERF-MycHis binding *in vivo*. Immunoprecipitation used anti-Myc (M), anti-FLAG (F) or no antibody (–). Amplification of immunoprecipitates alongside corresponding input DNAs used the same primer pairs as were employed to generate the corresponding fragments for EMSA (see Supplementary Table 1), Samples were from Flp-In^{TM} T-REx^{TM}-293 cells induced for mTERF-MycHis expression, except for fragment Leu, where extracts from uninduced cells were also tested.

Induction of mTERF-MycHis expression enabled immunoprecipitation of several key fragments of the mitochondrial genome in which binding was found *in vitro* (Figure 2d) The fragment containing the canonical mTERF-binding site in the $tRNA^{Leu(UUR)}$ gene was routinely detected in the anti-Myc immunoprecipitate from induced cells, but was not immunoprecipitated by control antibody (anti-FLAG) or no antibody. Immunoprecipitates from uninduced cells were negative under comparable conditions, but using excess anti-Myc antibody we sometimes observed weak amplification of this fragment (data not shown), consistent with a low level of leaky expression of the mTERF-MycHis transgene and the high affinity of the protein for the canonical binding site. Consistent positive signals were also seen in the anti-Myc immunoprecipitate from induced cells, but not control immunoprecipitates, for the HSP-containing fragment OH5 and for the three D-loop fragments (NCR1, NCR5 and OH1) which gave the strongest EMSA signals *in vitro* (Figure 2c). The ND1.1 fragment internal to the ND1 coding sequence was also weakly amplified from anti-Myc immunoprecipitates from induced cells (Figure 2d). Fragments from the ND3 gene (e.g. ND3.4), or others which were negative for binding *in vitro* using EMSA, gave either very faint signals or no signal at all after immunoprecipitation. Overall, these findings are consistent with the proposition that mTERF, when overexpressed, can bind *in vivo* to specific, non-canonical binding sites, which correspond with binding sites detected *in vitro*.

Replication pause sites map close to sites of mTERF binding in human mtDNA

In previous studies using 2DNAGE we noted the occurrence of a number of stereotypic pause sites in mitochondrial DNA of both sea urchins (38) and vertebrates (21,24). In sea urchins, pause sites correspond with sites of specific protein binding (39–41). We therefore considered the hypothesis that some of the replication pause sites in human mtDNA may map to locations of mTERF binding.

We initially analysed the region of the genome in which the canonical mTERF-binding site in the $tRNA^{Leu(UUR)}$ gene is located. 2DNAGE analysis of the PvuII–AccI fragment covering this site, extending from O_L into the rDNA, in several different cell lines and tissues (Figure 3), revealed a number of pause sites of varying prominence. To visualize their positions more clearly we treated parallel samples with S1 nuclease, thus digesting partially degraded RITOLS intermediates, including any attached RNA tails. The two epithelia-derived cell-lines, HEK293T and HeLa, gave very similar patterns, with a clear, though relatively weak pause site signal in the region of the $tRNA^{Leu(UUR)}$ gene (designated 'a' in Figure 3b), a second, more prominent pause located in the 3′ part of the ND1 gene or in the adjacent IQM tRNA cluster (designated 'b'), a third, near O_L (designated 'd'), and accumulated material in a broad region of ND2 (designated 'c').

In 143B (osteosarcoma) and Jurkat (T-cell leukaemia) cells the steady-state abundance of all mtDNA RIs was quantitatively less, though the patterns were qualitatively similar to those seen in HEK293T or HeLa cells. In S1-untreated material the pause sites were poorly resolved, and the descending segment of the Y-arc was very weak. Region 'c' was not seen as a discrete species, even after S1 treatment. Following S1 treatment, the ratio of the other pauses differed between cell-types: for example, pause 'b' was much more prominent than pause 'd' in Jurkat cells, whereas in 143B cells they were at similar abundance. The $tRNA^{Leu(UUR)}$ gene pause 'a' was seen clearly in all cell-lines tested.

In tissue samples (Figure 3c), pause 'a', near to the canonical mTERF-binding site, was most prominent in the brain, but weak in other tissues tested. Pause 'd' was more prominent than pause 'b' in heart and brain, but weaker than pause 'b' in skeletal muscle and in kidney. Pause 'c', was seen only in kidney, where both it (and pause 'b') appeared to be even more diffuse than in other tissues or cell-lines. An additional pause site was seen in brain, between 'c' and 'd' (denoted 'd'). Pause 'd', near O_L, was also detected as an extended pause region in human placenta [(24), Supplementary Figure 3].

Pausing near two other sites at which mTERF binding to DNA was seen both *in vitro* and *in vivo* (Figure 2), namely O_H and the TAS region, is already well documented from previous studies, and further examples are seen in Figure 4 (see also Supplementary Figures 3 and 4). Although originally proposed as a unique unidirectional origin, recent data indicate that O_H also functions as a site of fork arrest when bidirectional replication initiates elsewhere, and may thus also be considered as the terminus of replication [(20–22,24), see also Supplementary Figure 3]. The TAS region is, by definition, adjacent to the termination site for the synthesis of D-loop 7S DNA.

mTERF overexpression enhances replication pausing in human mtDNA

In order to test whether the level of mTERF expression influences replication pausing we carried out 2DNAGE

(**E**) Summary of EMSA results combining the data from this figure, Supplementary Figure 2, and other (negative) data not shown. The regions of the genome which were probed are reproduced from part (a) of the two figures, plus the ND5-ND6 gene junction which was probed using a dsDNA oligonucleotide. Binding is denoted as strong (filled circles), moderate (grey circles), weak (open circles), questionable (dotted circle, fragment OH5, as discussed in the text and legend to Supplementary Figure 2) or absent (no circles). Our inference of binding is based on the fact that EMSA signals were enhanced by induction of expression of both mTERF and mTERF-MycHis, that the complexes migrated at slightly different positions consistent with the presence of the epitope tag in the latter case, and that the complexes formed by mTERF-MycHis were supershifted by the anti-Myc antibody. The assertion that binding is strong, moderate or weak is based either on actual competition experiments (OH1 and ND1.1), or simply on the strength of the EMSA signal. Where the above criteria were not fulfilled, binding was scored as negative. In summary, the ND1 coding region and following IQM tRNA cluster contain at least four weak binding sites for mTERF. The NCR contains three weak binding sites, as well as three additional sites which showed very weak or questionable mTERF binding, as shown. A weak binding site was also found in the vicinity of O_L.

Table 1. SELEX analysis of the mTERF-binding site

Position	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Occurrences[a]														
A	0	0	0	3	30	17	23	21	1	0	0	0	0	2
C	0	0	0	7	4	6	1	3	0	0	15	0	0	0
G	8	43	43	2	4	16	6	16	31	0	0	43	43	0
T	35	0	0	31	5	4	13	3	11	43	28	0	0	41
Consensus[b]	t	G	G	t	a	r	a	r	g	T	Y	G	G	t

[a]Out of 43 clones analysed which matched a clear consensus (see text).
[b]Nucleotides found in 43/43 clones shown in upper case, others in lower case, Y = pyrimidine, R = purine.

analysis of RIs in mtDNA extracted from cells induced to overexpress mTERF, compared with uninduced cells (Figure 4, Supplementary Figure 4). Within the 3.6 kb PvuII–AccI ND2-containing fragment (Figure 4b), pause site 'a' (tRNA$^{Leu(UUR)}$, mapping near the canonical site of mTERF binding) was strongly enhanced by mTERF overexpression, compared with the unit-length restriction fragment (denoted 1n in Figure 3d). Pause sites 'b' (ND1/IQM tRNA cluster) and 'd' (O_L) were also enhanced, as was the more diffuse pause region 'c'. In mTERF-overexpressing cells we also detected a more prominent X-form intermediate (designated 'x' in Figure 4b, Supplementary Figure 4a) in restriction fragments (e.g. HincII or AccI) containing the tRNA$^{Leu(UUR)}$ gene at a central location.

Within the NCR mTERF overexpression enhanced the abundance of a paused intermediate migrating near or beneath the bubble arc (designated 'f' in the HincII fragment and 'n' in the AccI fragment, O_H probe), as well as the arc leading to it from the unit-length fragment (Figure 4c, Supplementary Figure 4b). These forms were sensitive to S1 nuclease (Supplementary Figure 4b) and are probably equivalent to the classical D-loop. mTERF overexpression also appeared to increase the relative amount of 7S DNA as well as introducing subtle alterations to the various forms of mtDNA resolved on 1D gels (Supplementary Figure 4f). mTERF overexpression also diminished the relative abundance of termination intermediates (designated 't' in Figure 4c and d) and increased that of Y-form intermediates in which a single fork appears to have paused when approaching O_H (designated 'g'). The distribution of material on the termination arc also appeared to be subtly different from that of uninduced cells.

In other regions, a prominent pause site ('h', Figure 4c), located near to the ND5/ND6 gene boundary, was unaffected by mTERF overexpression, whereas a novel pause was induced in the coding region of ND3 (Supplementary Figure 4c). Note, however, that strong mTERF binding was not found *in vitro* in either region (Supplementary Figure 2 and other data not shown).

Digestion of mtDNA from mTERF-overexpressing cells with restriction enzymes having only a single recognition sequence in the genome generated 2DNAGE patterns consistent with enhanced pausing in the region of ND1/tRNA$^{Leu(UUR)}$ and consequent delayed resolution in the NCR (Figure 4d, Supplementary Figure 4d and e). Note that mTERF overexpression produced subtle, site-specific effects, rather than a general slowing of replication e.g. as would be attributable due to non-specific stalling.

mTERF knockdown diminishes replication pausing in the ND1/tRNA$^{Leu(UUR)}$ region

To test whether the modulation of replication pausing resulting from mTERF overexpression represents the signature of a finely tuned physiological process rather than just an overexpression artefact, we downregulated the expression of mTERF by RNA interference. This produced a reciprocal effect on mtDNA replication pausing at the canonical mTERF-binding site. We first tested several different mTERF-directed siRNAs in transient transfection assays, using cells stably transfected with the mTERF-MycHis expression construct, enabling us to evaluate knockdown at the protein level by western blotting (Figure 5a). One particular siRNA (mTERF.1) gave consistently strong knockdown, as judged also by immunocytochemistry on mTERF-MycHis-expressing cells (Figure 5b) and EMSA (Figure 5c). Based crudely on the autoradiographic EMSA signals, functional knockdown of >90% was routinely achieved 48 h after transfection with siRNA mTERF.1. The effects of mTERF knockdown on replication pausing in the ND1/tRNA$^{Leu(UUR)}$ region were then studied using 2DNAGE (Figure 5d). Pause site 'a' (tRNA$^{Leu(UUR)}$) was no longer detectable, even on long autoradiographic exposure, and the prominence of X-forms was also diminished by mTERF knockdown. The abundance of other pauses was altered less substantially, though the relative amount of species 'b' compared with 'c' appeared to be decreased.

mTERF overexpression enhances lagging strand 5′ ends near to specific replication pause sites

One signature of increased replication pausing during strand-coupled DNA replication should be the enhancement of persistent, lagging strand 5′ ends adjacent to pause sites (Supplementary Figure 5). We used LM-PCR to map such ends in the vicinity of the major pauses regulated by mTERF, and to determine the effects upon them of mTERF overexpression. Comparing mtDNA from cells overexpressing mTERF with that from uninduced cells, we analysed 5′ ends on the L-strand near the canonical tRNA$^{Leu(UUR)}$ binding site, as well as in the whole of ND1 and the adjacent tRNA genes, and also on the H-strand in the NCR. A cluster of L-strand 5′ ends in or adjacent to the tRNA$^{Leu(UUR)}$ gene, notably at np 3211,

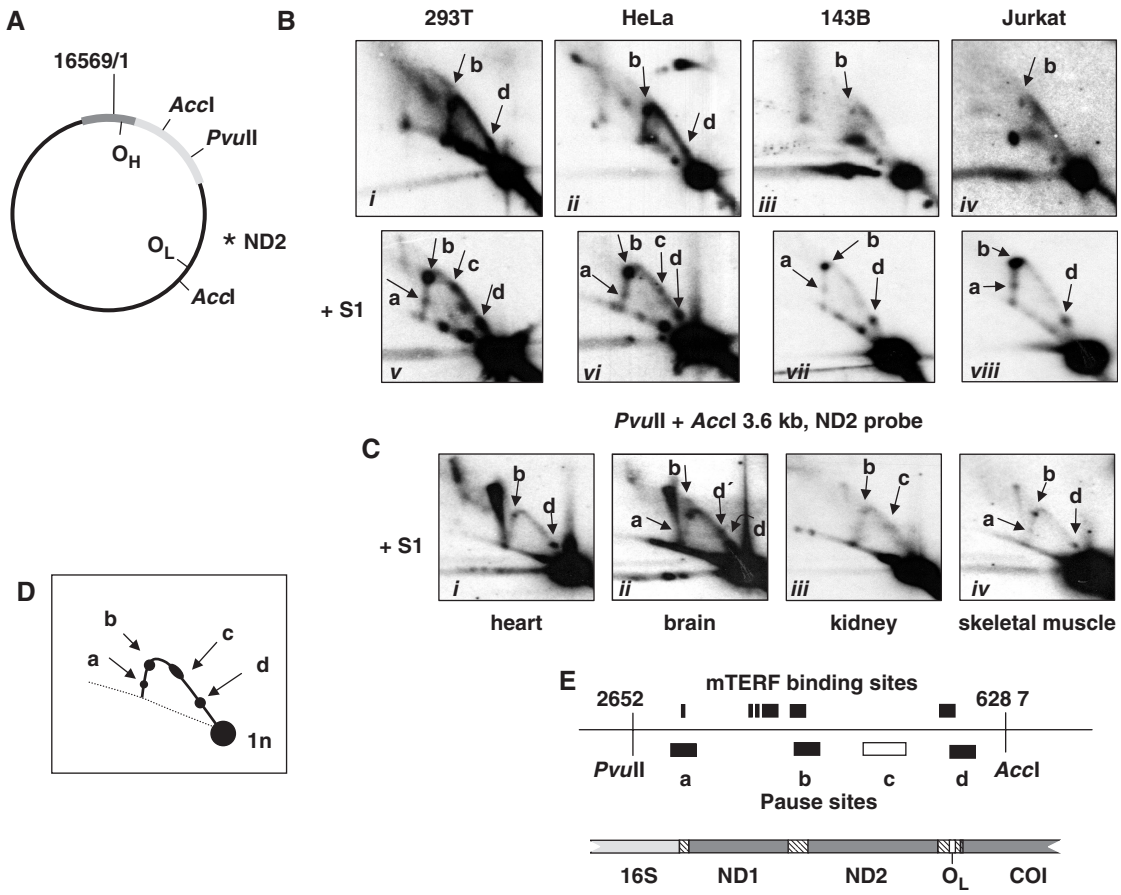

Figure 3. 2DNAGE analysis of replication pausing in human mtDNA (region spanning from 16S rDNA through O_L). (**A**) Schematic map of human mtDNA showing relevant restriction sites, O_H, O_L, the approximate locations of the probe used (ND2, see Supplementary Table 1), denoted by an asterisk, the NCR (bold, dark grey) and rDNA (bold, pale grey). (**B**) 2DNAGE analysis of ND2-containing (3.6 kb) PvuII–AccI fragment from four human cell-lines as indicated, with and without treatment, after digestion, with S1 nuclease. Prominent pause sites 'a'–'d', arrowed, schematized in part (d). A prominent intermediate, lying below the Y-arc between 'c' and 'd', is a product of S1 nuclease digestion, and probably represents a paused species containing a single-stranded gap. (**C**) 2DNAGE analysis of the 3.6 kb PvuII–AccI fragment from four human autopsy tissues as indicated, treated with S1 nuclease after digestion. Prominent pause sites 'a'–'d', arrowed, schematized in part (**D**). (**E**) Map of the 3.6 kb PvuII–AccI fragment showing the approximate locations of the pause sites and mTERF-binding sites, with the gene locations in the region (16S rRNA in light grey, ND1, ND2 and COI protein-coding genes in dark grey, tRNAs cross-hatched, non-coding DNA at O_L in white) shown below. Limits of the mTERF-binding sites are based on the EMSA data of Figure 2 and Supplementary Figure 2. Approximate locations of pause sites extrapolated from first-dimension migration, calibrated by positions of 1n, 2n, linear partials and apex of Y-arc. Depending on compression artefacts, actual pause sites may be located up to 150 bp further from the ends of the segments as shown. Pause 'c' (open box), when present, appears to extend over a wider segment than the other pauses.

3234 and 3310, were strongly enhanced during 72 h of induction of mTERF overexpression (Figure 6a, Supplementary Figure 5b). In the vicinity of the IQM tRNA gene cluster, LM-PCR revealed L-strand 5′ ends enhanced by mTERF overexpression at np 4476 (close to the 5′ end of ND2 mRNA) and np 4434 (within the $tRNA^{Met}$ gene), against a background of heterogeneous 5′ ends that were generally unaffected by mTERF overexpression (Figure 6b and c). The significance of this heterogeneous background of abundant 5′ ends is unclear, although the two sites enhanced by mTERF overexpression lie in the vicinity of pause 'b'. Within the remainder of the ND1 and ND2 coding regions (Supplementary Figure 5c–h) we detected only weak LM-PCR signals which were not affected by mTERF overexpression. The prominent 5′ ends in the $tRNA^{Cys}$ gene adjacent to O_L were also unaffected by mTERF overexpression.

In the NCR, H-strand 5′ ends at O_H, as well as those clustered in the distal region of the D-loop (np 16 311, 16 337, 16 370, 16 411 and more weakly at np 16 197) were strongly induced by mTERF overexpression. This is consistent with delayed resolution, arising from more frequent pausing at the canonical mTERF-binding site. H-strand 5′ ends in the NCR are on the lagging strand for initiation events outside of the NCR, and thus may also be enhanced by pausing of replication forks entering the NCR from the 'cytochrome *b* side'.

DISCUSSION

mTERF is a modulator of replication as well as transcription

In this study, we showed that mTERF binding at its canonical binding site in the $tRNA^{Leu(UUR)}$ gene

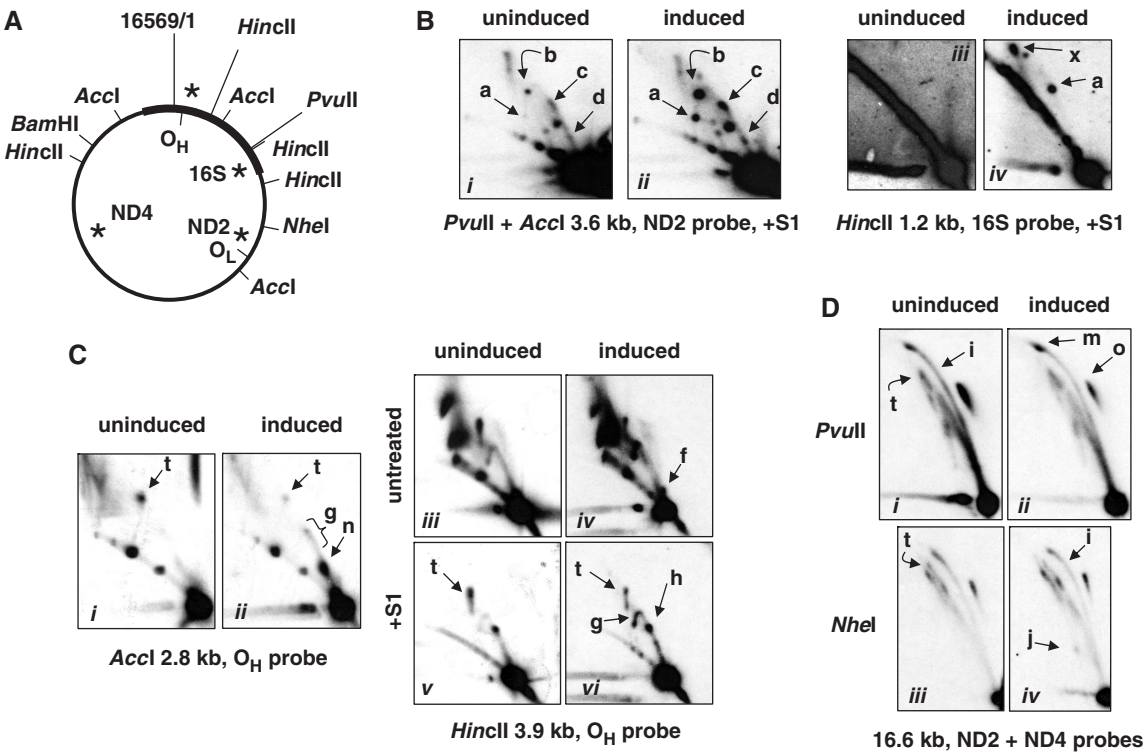

Figure 4. 2DNAGE analysis of mtDNA replication pausing in mTERF-overexpressing cells. (**A**) Schematic map of human mtDNA using same nomenclature as Figure 3a, showing relevant restriction sites and approximate locations of probes. (**B–D**) 2DNAGE analysis of mtDNA from cells induced to overexpress mTERF, compared with uninduced cells. To facilitate visualization of pause sites, samples were treated with S1 nuclease following restriction digestion, where indicated. Pause sites 'a'–'d' arrowed, plus other species as discussed in text. The exposures of the uninduced and induced panels *i* and *ii* of (b) were adjusted for comparability, based on loading, after measuring phosphorimager signals from the unit-length fragment. Pause site 'h', as visualized in part (c), appears to be unrelated to mTERF expression. In part (d), the paused bubble, 'm' (PvuII digest), and the highly asymmetric double-Y species 'j' (NheI digest), both enhanced relative to other species by mTERF overexpression, are predicted products of pausing in the ND1/tRNA$^{Leu(UUR)}$ region. Subtle modifications are also seen in the region of the gel in which X, double-Y and broken theta molecules migrate, notably a decrease in the abundance of putative termination intermediates 't' (see also Supplementary Figure 4). 'o'—uncut circles and gapped circles.

influences replication pausing near to this site. Overexpression of mTERF enhanced this pausing and increased the steady-state abundance of lagging strand 5′ ends adjacent to the binding site, whereas mTERF knockdown by RNA interferences decreased pausing in the tRNA$^{Leu(UUR)}$ gene region. In addition, based on studies *in vitro* (EMSA, SELEX) and supported by findings *in vivo* (mIP), we identified novel sites of mTERF binding, elsewhere in the genome. Binding at these sites was weaker than in the tRNA$^{Leu(UUR)}$ gene, but replication pausing in these regions was nevertheless influenced by mTERF overexpression. The data support a role for mTERF as a modulator of replication, especially at its canonical binding site. Close parallels with the properties of replication pause-region binding proteins in bacteria and eukaryotic nuclei provide some intriguing hypotheses which we now discuss.

mTERF binding to additional sites in human mtDNA

The present study revealed novel sites of mTERF binding in the NCR and ND1 gene. mIP assays were consistent with binding at least at some of these sites by over-expressed mTERF *in vivo*. mTERF homologues in invertebrates, such as DmTTF in *Drosophila* (12) or DBP in sea urchins (39,41), also have diverse and multiple binding sites, typically demarcating the 3′ ends of oppositely transcribed gene clusters. Although mTERF binding to the novel binding sites appeared weaker than at the canonical binding site, many of them are clustered, suggesting that cooperative binding might promote site occupancy *in vivo*, consistent with the results of mIP. The binding we observed *in vivo* might also depend on other mitochondrial nucleoid proteins, including TFAM, as well as possible post-translational modifications.

The effects of mTERF overexpression on mtDNA RIs from the NCR (Figure 4c and d), suggest that mTERF may interact with this region *in vivo* to promote fork arrest at the replication terminus. The termination zone for mtDNA replication appears not to be a single point (O_H), but an extended region of the NCR (Figure 4c). mTERF overexpression resulted in increased stalling of replication forks as they approach O_H from the 'cytochrome *b* side'. It also appeared to elevate the abundance of 7S DNA (Supplementary Figure 4f) and of S1 nuclease-sensitive species probably equivalent to the classical D-loop (Figure 4c), consistent with increased pausing in the TAS region. Protein-binding sites within the TAS region were previously mapped by *in vivo* footprinting (42) and by EMSA (43), and mTERF might be one of the

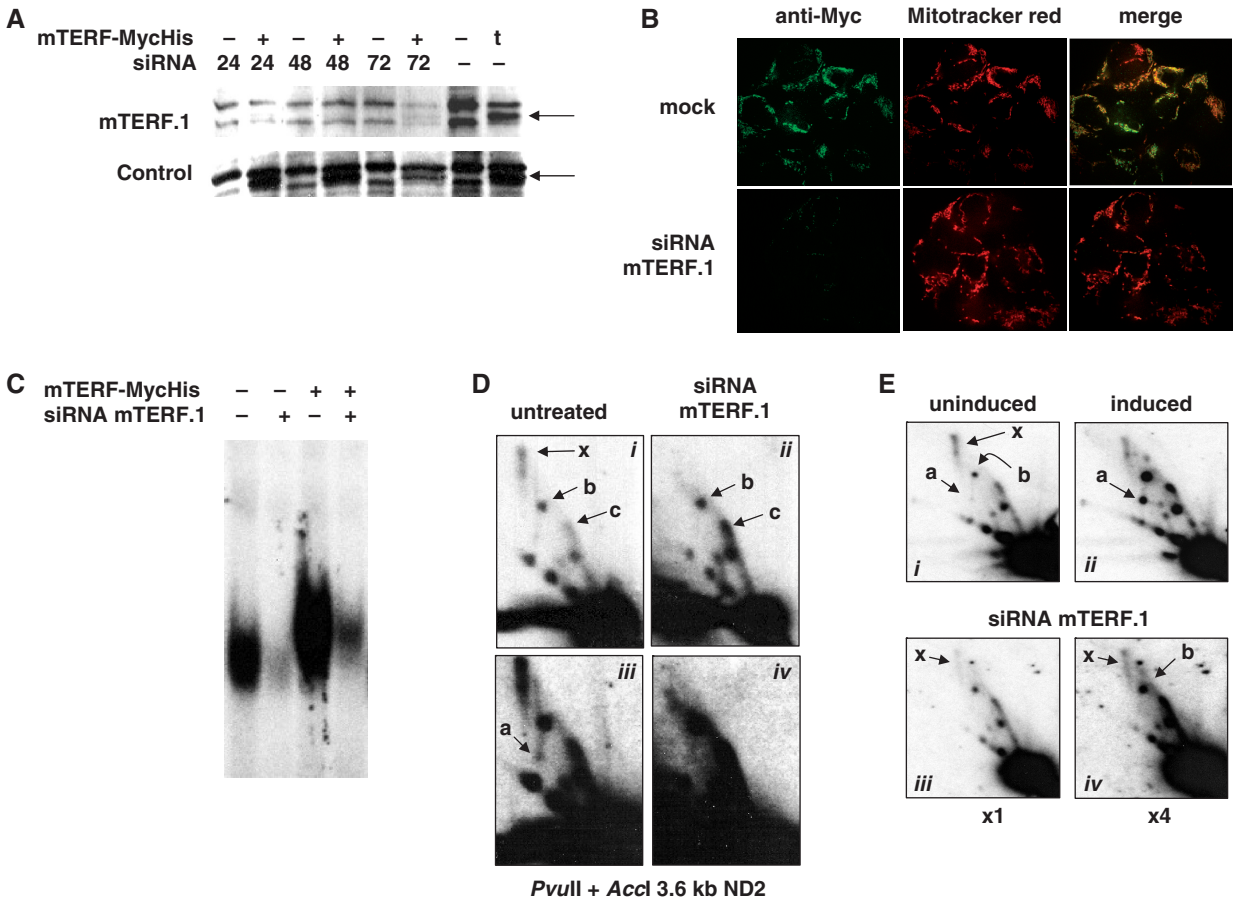

Figure 5. Downregulation of mTERF expression by RNA interference. (**A**) Western blot assay of mTERF knockdown by siRNA mTERF.1 (directed against mTERF mRNA) and siRNA Control. HEK293T cells were either untransfected (−), transiently transfected (t) or stably (+) transfected with an mTERF-MycHis expression construct. Cells were then assayed 24, 48 and 72 h following siRNA transfection or else without such transfection (−). The arrowed band is the mTERF-MycHis fusion protein, migrating between two background bands which appear in all westerns and thus provide an internal loading control. Note that the sample from untransfected, non-siRNA-treated cells in the upper panel (penultimate lane) is approximately 3-fold overloaded. (**B**) Immunocytochemistry of HEK293T cells stably transfected with mTERF-MycHis expression construct and then either mock transfected or transiently transfected with siRNA mTERF.1. Immunocytochemistry used the anti-Myc monoclonal antibody, and counterstaining with Mitotracker Red. (**C**) EMSA using Leu-short dsDNA oligonucleotide probe and mitochondrial protein extracts from HEK293T cells with or without stable transfection of mTERF-MycHis expression construct, followed by transient transfection for 48 h with or without siRNA mTERF.1. Despite the apparent difference in signal, the experimental conditions are the same as in Figure 1c: only the exposure time is different, and the amount of background signal in the gel. (**D**) 2DNAGE of mtDNA from untreated HEK293T cells or cells transfected with siRNA mTERF.1 for 48 h. *Pvu*II + *Acc*I digest (S1 treated) probed for the 3.6 kb fragment using ND2 probe. Panels *iii* and *iv* are longer exposures of panels *i* and *ii*, respectively. Note the down-regulation of the X-spike ('x') and pause site 'a', as well as of pause site 'b' relative to pause region 'c' (see Figure 3). (**E**) Phosphorimager-calibrated exposures of 2DNAGE blots from siRNA-treated cells (panel *iii*) alongside the corresponding images (panels *i* and *ii*) from uninduced and induced mTERF over-expressing cells, reproduced from Figure 4b. A longer exposure (panel *iv*) confirms the absence of pause 'a'.

proteins involved. The D-loop remains enigmatic. DNA synthesis arrest at TAS might be a switching mechanism relevant to copy number control, or may have other purposes, such as mitochondrial nucleoid organization (44).

The *Escherichia coli* Tus protein, which regulates the termination of chromosomal DNA replication, may represent a useful paradigm for mTERF. Tus binds multiple copies of the *Ter* sequence flanking the terminator region, and acts directionally as a contrahelicase (45) to trap replication forks in this region (46). The sea urchin mTERF homologue DBP has also been shown to function as a contrahelicase *in vitro* (15). Like Tus, mTERF binding to sites on both sides of the replication terminus region might regulate the entry of oppositely moving replication forks into the region, facilitating their orderly synopsis. Increased mTERF expression resulted in elevated levels of persistent H-strand 5′ ends in the NCR (Figure 6d), an expected signature of delayed resolution if fork passage through rDNA is more restricted. The orientation of potential mTERF-binding sites in the genome appears highly non-random. Taking the simplified sequence $GG(N_8)GG$ as the minimal binding site, its 12 occurrences in the NCR all bear the same orientation. The same applies to the cluster of seven such sites in the 3′ portion of ND1 and the adjacent tRNA gene cluster. In contrast, the canonical binding site shows the opposite orientation, although is flanked on each side by two oppositely oriented copies of the minimal binding site (Supplementary Figure 6).

Although we detected only ambiguous mTERF binding *in vitro* in the H-strand transcriptional initiator region,

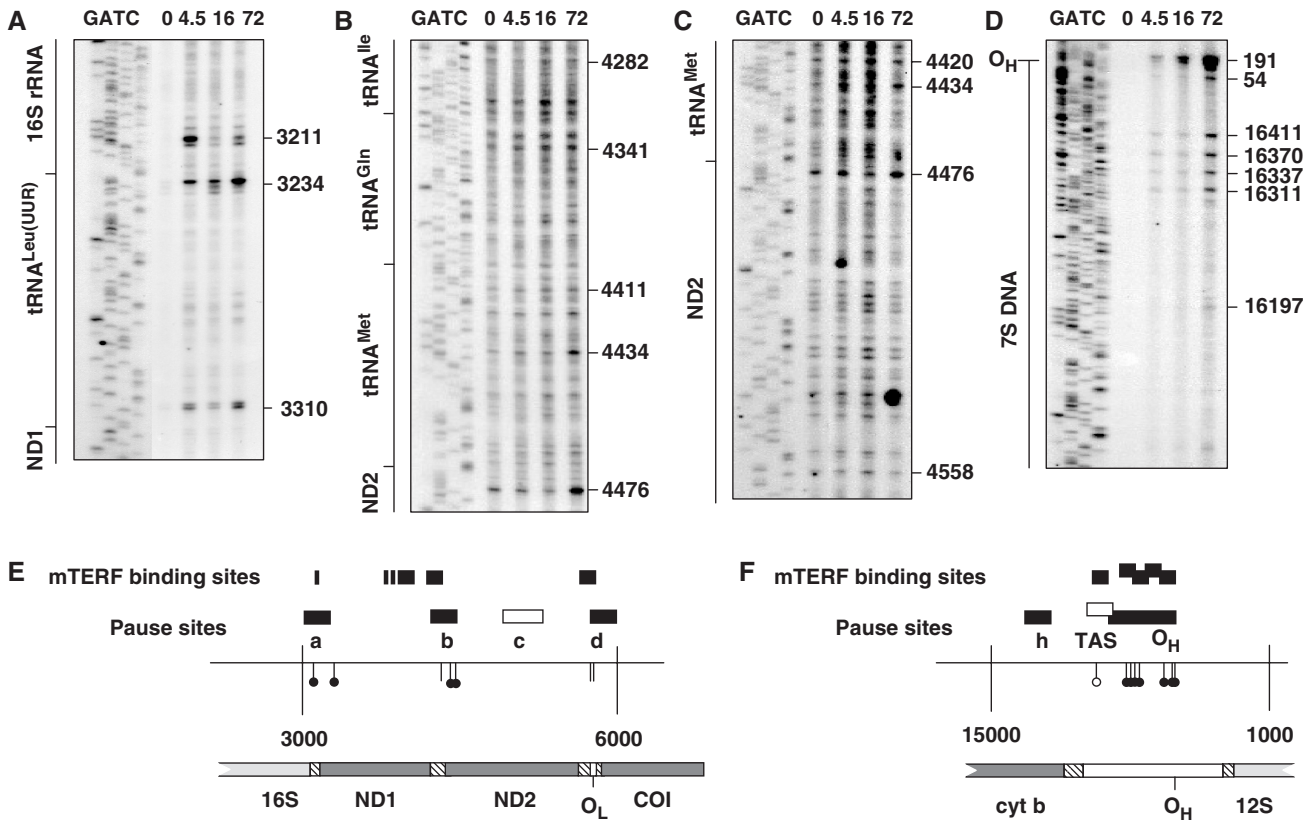

Figure 6. LM-PCR analysis of DNA 5′ ends. (**A**–**D**) Analysis of L-strand 5′ ends in the ND1-ND2 region, using primer sets TL1/TL2/TL7, TL8/TL9/L11 and TL8/TL9/TL11, respectively, shown alongside sequencing ladders for the corresponding segments. Samples analysed represent a time-course of induction of mTERF overexpression from 0–72 h (d). Analysis of H-strand 5′ ends in the O_H region using primer set H1/H2/H5. (**E**) and (**F**) Schematic summary diagrams of the LM-PCR findings in the 16S rRNA-O_L and O_H regions, respectively. Shown below the scale lines are the positions (vertical lines) of the major 5′ ends detected, with those exhibiting clearly increased abundance in mTERF-overexpressing cells also indicated by filled circles. Gene locations shown below (12S and 16S rRNA in light grey, cyt b, ND1, ND2 and COI protein-coding genes in dark grey, tRNAs cross-hatched, non-coding DNA in NCR and at O_L in white). LM-PCR data are compiled from parts (a–d) of this figure, plus parts (b–i) of Supplementary Figure 5. Above the scale lines are indicated the positions of mTERF-binding sites and replication pauses inferred from other experiments: in (e) reproduced from Figure 3d, and in (f) compiled from data of Figures 2 and 4, plus Supplementary Figure 2. Based on the data of Figure 4, the O_H pause region enhanced by mTERF overexpression extends across most of the NCR. The white box in (f) indicates the assumed position of the pause site giving rise to species 'n' in Figure 4c, i.e. assuming initiation close to O_H. The open circle denotes the minor lagging strand 5′ end mapping in this region (np 16197), which was enhanced by mTERF overexpression. The positions of the various mTERF-binding sites in the NCR were inferred from EMSA using overlapping 150 bp fragments, as shown.

(Supplementary Figure 2e, EMSA probe OH5), mIP findings suggest that the site is efficiently bound *in vivo* by overexpressed mTERF. The latter is consistent with previous findings that recombinant mTERF (7,29) binds only weakly to this site, whereas partially purified, endogenous mTERF binds more strongly and establishes a DNA loop required for efficient rDNA transcription (7). This may require a post-translational modification or limiting accessory factor found only *in vivo*. mTERF exists in multiple isoforms with distinct properties (4,47), and has been reported to convert to an inactive trimeric form *in vitro* (48). The initiator fragment also contains binding sites for TFAM, which may promote binding but may also interfere with the interpretation of the EMSA assay (see Supplementary Figure 2e).

Regulated passage of replication and transcription complexes

The role of mTERF as a transcriptional terminator is well established from *in vitro* studies, and DmTTF also functions thus *in vivo* in *Drosophila* (13). However, there is no compelling evidence that mTERF regulates mitochondrial RNA levels physiologically. The disparity in relative abundance between mRNAs and rRNAs in mammalian mitochondria can largely be accounted for by post-transcriptional regulation, notably differences in half-life (49) and RNA processing efficiency (50). Despite causing reduced mTERF-binding affinity and terminator activity *in vitro*, the 3243A>G MELAS mutation has almost no effect on mitochondrial RNA levels *in vivo* (5). Moreover, manipulation of mTERF levels *in vivo* by overexpression or RNA interference has remarkably little effect on steady-state mitochondrial RNA levels (Hyvärinen *et al.*, manuscript in preparation). The observation that mTERF also modulates mtDNA replication pausing suggests a different physiological meaning for its action as a transcriptional terminator, i.e. it coregulates replication and transcription.

The unregulated collision of oppositely moving transcription and replication complexes drastically inhibits DNA replication and provokes genomic instability in both bacteria (51,52) and yeast (53,54). In *E. coli*, head-on collision of the transcription and replication machineries severely impedes the progress of the replication fork (51) whereas codirectional transcription has no effect. Within the *E. coli* chromosome almost all essential genes are oriented such that transcription and replication are codirectional (55), which is proposed to minimize the mutagenic effect of repeated replication stalling and recombinational restart, following head-on collisions (51,56). In bacterial or yeast plasmids, or yeast rDNA, such head-on collisions can trigger genomic instability (53,57,58), e.g. due to knotting of daughter duplexes (59). In mammalian nuclei, head-on collisions can trigger the formation of HSRs (homogeneously staining regions of chromosomes), the signature of massive gene amplification events (60).

Proteins with dual roles in replication and transcriptional arrest are well documented. The *E. coli* Tus protein, described in the preceding section, preferentially blocks transcription with a similar polarity as DNA replication (61,62). Passage of a transcription complex from the permissive direction relieves the block on DNA synthesis (61) by provoking the dissociation of bound Tus (63). The mouse TTF-I protein binds at the 3′ end of the rDNA transcription unit, where it terminates transcription by RNA polymerase I (64) and arrests replication forks arriving from the other direction (65,66), via its polar contrahelicase activity (67). This organization of the rDNA locus is relatively conserved throughout eukaryotes, although in some species the TTF-I homologue co-operates with or depends upon other proteins to maintain the replication fork barrier (RFB), including Sap1p and Reb1p in *Schizosaccharomyces pombe* (68–70), with involvement of Swi1p and Swi3p to stabilize the stalled forks (71), or Fob1p in *Saccharomyces cerevisiae* (72,73), with Sir2p regulating recombination at the stalled forks (74).

The entry of replication forks into the mtDNA termination zone around O_H requires traversal of the heavily transcribed rDNA region in the antisense direction, with potentially catastrophic consequences if a transcription complex is encountered. By binding at the rDNA boundary, mTERF may thus serve a function related to those of both Tus and TTF-I, facilitating the regulated passage of oppositely moving transcription and replication machineries, and regulating fork access to the termination zone.

The passage of a transcriptional complex in the permissive direction may also serve a regulatory role, such as hypothesized for mtDBP in relieving the block on D-loop expansion in sea urchins (15).

mTERF overexpression enhanced X-like species in fragments where the canonical mTERF-binding site was centrally located (Figure 4b, Supplementary Figure 4a), whereas mTERF knockdown by RNA interference depleted such species (Figure 5d). Although these may be recombination intermediates (see below), they might also comprise termination complexes centred on the mTERF-binding site. If increased mTERF activity enhances rDNA transcription (6), it may also restrict the entry of replication forks into rDNA in the antisense direction and perhaps even shift the resolution site in some molecules from the NCR to the $tRNA^{Leu(UUR)}$ gene. However, this must be a very minor fraction of molecules, since we did not see a complete Y-arc in O_H-containing fragments.

Transcription termination and the bootlace model

Previous studies of vertebrate mtDNA replication indicated that, in the majority of molecules, the lagging strand is initially laid down in the form of extended RNA segments which are subsequently converted to DNA via a maturation step (19). We hypothesized that the RNA lagging-strand may arise via either of two highly unorthodox mechanisms: either via a primase capable of synthesizing extended RNA primers, or by the hybridization of preformed L-strand RNA with the displaced H-strand in a 3′ to 5′ direction as the replication fork advances, the so-called bootlace model.

mTERF-dependent replication pausing may be construed as circumstantial evidence supporting the bootlace model. Transcriptional termination by mTERF adjacent to a paused replication complex would provide a 3′ end capable of priming lagging-strand DNA synthesis, at the same time as delivering a fresh RNA bootlace to enable the replication fork to proceed in the forward direction. The mTERF-dependent enhancement of lagging strand 5′ DNA ends near the canonical (and some other) mTERF-binding sites, (Figure 6), supports this idea. In sea urchin mtDNA, the major replication pause-region, which interacts with at least two DNA-binding proteins (38,40,41), also appears to be a major lagging-strand origin, as well as a site of transcriptional termination and/or RNA processing.

The effect of overexpression of mTERF is much more site-specific than that produced by overexpression of TFAM (28), by treatment with mtDNA replication inhibitors such as dideoxycytidine (28), or by expression of dominant-negative versions of the mtDNA helicase Twinkle (31). Unlike these treatments, mTERF overexpression did not cause a general slowing of replication, and did not alter globally the ratio of strand-coupled and RITOLS type RIs. By facilitating lagging-strand maturation, mTERF may serve merely to minimize the extent of the region of mtDNA maintained in the more vulnerable RNA-DNA hybrid form, thus contributing to genome stability.

Recombination at the mTERF-binding site

An alternate interpretation of the X-like molecules centred on the $tRNA^{Leu(UUR)}$ gene region, which were enhanced by mTERF overexpression and depleted by mTERF knockdown, is that they represent true recombination intermediates. Such forms would be expected to arise if persistent mTERF binding and consequent prolonged pausing entrain fork collapse, requiring either a double-strand break or fork regression to generate a recombinogenic end for restart of replication. We previously

noted that the canonical mTERF-binding site was a frequent break-point in rearranged mtDNA molecules ('sublimons') detectable at a low level in all cell-types, but especially prominent in human heart (75), and also in mice expressing a disease-equivalent version of the Twinkle helicase(76) associated with autosomal dominant external ophthalmoplegia (PEO). It is tempting to ascribe such molecules to aberrant recombination following pausing and fork collapse at the $tRNA^{Leu(UUR)}$ gene.

In yeast, double-strand breaks at the site of the RFB in rDNA, giving rise to low-level genomic rearrangements implicated in ageing, are evident even in wild-type strains (54). However, in strains defective for the DNA helicase Rrm3p, which is required for the processing of paused replication forks at sites of protein binding (77,78), the frequency of such events is greatly increased. If the balance between the pause-inducing and pause-processing machineries is disturbed, recombinational mechanisms must be employed to restart replication, with the concomitant risk of genomic instability. Thus, even though replication pausing systems such as mTERF may have evolved to limit genomic instability by preventing collisions of the replication and transcription machineries, their dysregulation, including by overexpression, could itself lead to instability. It follows that mTERF is a candidate gene for involvement in those cases of genetic disorders mediated by mtDNA rearrangements (e.g. PEO), whose genetic basis has not yet been elucidated.

In some cases replication pausing is merely a signature of defective replication (79,80). In others it is clearly a programmed event which facilitates other processes and preserves genome stability (81). As indicated by our findings (Figure 3), the phenomenon of pausing in human mtDNA is not confined to just one cell-type, nor is it an *in vitro* artefact seen only in cultured cells. The fact that it exhibits differences between cell-types and tissues strengthens the proposition that it is of physiological significance. The involvement of mTERF in modulating mtDNA replication pausing in human mtDNA, and the analogies with programmed replication pausing in other systems, support the idea that mTERF represents a system for safeguarding the integrity of the mitochondrial genome, whilst facilitating its efficient expression.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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The mitochondrial transcription termination factor mTERF modulates replication pausing in human mitochondrial DNA

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SUPPLEMENTARY DATA

LEGENDS TO SUPPLEMENTARY FIGURES

Supplementary Figure 1

Effects of overexpression of mTERF on mtDNA copy number and sequence-specific protein binding activity. (a) Q-PCR analysis of mtDNA copy number of Flp-In™ T-REx™-293 cells transfected with the mTERF construct and induced for the times indicated, normalized to the mean value for uninduced cells (means ± S.D. of 3 independently isolated DNA samples). The analysis compared the level of mtDNA (cytochrome *b* probe) with that of amyloid precursor protein (APP), as a single copy nuclear DNA standard, as previously (28). (b) EMSA supershift assay of protein binding to the $tRNA^{Leu(UUR)}$ gene-containing fragment Leu (see Fig. 2 and Supplementary Table 1) using mitochondrial protein extract from Flp-In™ T-REx™-293 cells, with (+) or without (–) induced overexpression of mTERF or the mTERF-MycHis fusion protein, as indicated. Supershift was carried out using anti-Myc (m) or anti-FLAG (f) antibodies as shown (see Materials and Methods). The supershifted complex formed using the anti-Myc antibody, but not the anti-FLAG antibody, is arrowed. This supershifting property makes it possible to distinguish complexes actually containing the over-expressed mTERF-MycHis. Note also the low mobility bands visible at this exposure in the left-hand panel, which are probably due to binding by TFAM. Similar complexes are revealed in the gel shown in the right-hand panel (mTERF-MycHis expressing cells), at longer (more comparable) exposures. Their appearance is dependent on the fragment tested and the autoradiographic exposure used, not on the expression of any transgene.

Supplementary Figure 2

EMSA analysis of mTERF binding. See Figure 2 of the main paper for other data. (a) Schematic diagram of regions of the mitochondrial genome in which binding was tested using 150 bp fragments, nomenclature as in Fig. 2a, with additional segments of protein-coding genes A8, A6, COIII, ND3 and ND4L as shown. The overlap between A8 and A6 is shown in black. [The additional regions analysed for binding were selected, based on the data of the main paper, as regions showing enhanced replication pausing in mTERF-overexpressing cells]. (b) Map showing overlapping, shorter fragments

used in EMSA to localize the binding sites within fragment ND1.1 (see below, part d). (c) Further detail of binding to 150 bp fragments shown in Fig. 2. Mitochondrial protein extracts were from cells induced (or not) to over-express natural mTERF, with or without 100-fold mass excess of cold competitor. (d) Localization of mTERF binding sites within fragment ND1.1 using shorter, overlapping fragments (see part b), and supershifting. Cold competitor was present, where indicated, in several-fold mass excess. The simplest interpretation is that there are two mTERF binding sites within ND1.1, one located in fragments ND1.12 and ND1.13 but absent from ND1.11 and ND1.16, i.e. lying between np 3830-3855, the second located within ND1.14 but absent from ND1.13, i.e. lying between np 3880-3907. Within each of these short segments a good match to the SELEX consensus is found (see Table 1). (e) EMSA analyses of binding to 150 bp probe fragments in addition to those shown in Fig. 2. Nomenclature as for Fig. 2. A very high molecular weight mTERF-dependent complex (arrowed) was formed by fragment OH5. Since fragment OH5 also contains an identified binding site for TFAM, this complex may contain TFAM as well as mTERF, and could correspond with the large complex visualized by Martin et al (6) at the initiator site for rDNA transcription. Other fragments gave no reproducible EMSA signals indicative of mTERF binding. The ND5-6 dsDNA oligonucleotide covering the ND5-ND6 gene junction (Supplementary Table 1, data not shown) also gave no signal.

Supplementary Figure 3

Examples of replication pausing near mTERF binding sites. (a) Schematic map of human mtDNA showing relevant restriction sites, O_H, O_L, the approximate locations of the probes used (O_H and COI, see Materials and Methods), denoted by asterisks, the NCR (bold, dark grey) and rDNA (bold, pale grey). (b) 2DNAGE analysis of O_H- and O_L-containing fragments from human placental mtDNA. Panels *i* and *ii* were treated, after digestion, with S1 nuclease and are images of gels already published in Ref. 24. Pause zones near O_H and O_L arrowed.

Supplementary Figure 4

Further 2DNAGE data showing effects of mTERF overexpression. (a) Comparable exposures of ND2-containing *Acc*I fragment, treated with S1 nuclease, in which pause site 'a' ($tRNA^{Leu(UUR)}$) is centrally located, showing enhancement of the species at the tip of the X-arc ('x'), as well as of the double-Y arc leading to it, in mtDNA from cells in which mTERF overexpression was induced for 48 h. (b) Longer exposure of panels *i* and *ii* from Fig. 4c, plus similar gels of material treated with S1 nuclease. Nomenclature as Fig. 4c. (c) *Bcl*I digest (treated with S1 nuclease), probed for the 3.3 kb ND3-containing fragment (ND4 probe). Novel pause in mTERF-overexpressing cells arrowed. (d) *Bam*HI digest probed as for the gels shown in Fig. 4d, using similar nomenclature. The steeply descending trajectory of the 'eyebrow' (broken-theta) arc, denoted 'e', is due to compression. i – initiation (bubble) arc, o – uncut circles, dY – double-Y molecules. Pausing in the ND1/$tRNA^{Leu(UUR)}$ region generates complex double-Y or broken-theta species in this digest (region of the gel designated 'l'), whose migration and interconversion depends on the timing of lagging-strand maturation to dsDNA at the *Bam*HI site. The enhanced pausing when mTERF is overexpressed is proposed to facilitate the more complete maturation of the lagging strand, such that the termination forms in the *Bam*HI digest are resolved mainly as double-Y molecules (in which the site is cut on both daughter duplexes), denoted 'u', as opposed to the more electrophoretically retarded broken-theta molecules, denoted 's', in which the site is cut only on one daughter branch, as seen in uninduced cells. (e) Illustration of the prominent intermediates generated by pausing in the ND1/$tRNA^{Leu(UUR)}$ region, which were detected in single-hitter digests (Fig. 4d and part (d) of this figure), as indicated. Filled and open ovals denote O_H and the ND1/$tRNA^{Leu(UUR)}$ pause region, respectively. (f) One-dimensional agarose gel-blot of undigested mtDNA, with or without heating to 95 ºC for 30 s to release 7S DNA (arrowed) by branch migration, as indicated. DNA was from cells induced for 24 h (ind) or not induced (unind) to overexpress mTERF. Lower panel indicates 7S DNA region of the gel (longer exposure). The upper panel indicates approximately equal loading and reveals subtle differences resulting from mTERF overexpression, in the molecular forms of mtDNA resolved on 1-D gels. Brackets indicate areas of the gel in which the migration of various forms of mtDNA from mTERF-overexpressing cells differed from those seen in uninduced cells.

Supplementary Figure 5

LM-PCR analysis of additional 5´ ends. (a) Schematic diagram indicating that sites of replication pausing, such as may result from a bound protein (filled oval), correspond also with persistent 5´ ends on the lagging strand. Nascent strands are shown with arrowheads to denote the 5´ to 3´ direction of synthesis. (b)-(i) LM-PCR reactions using the following primer sets: (b) TL1/TL2/TL7, (c) TL1/TL2/TL6, (d) TL1/TL2/TL5, (e) TL1/TL2/TL4, (f) TL1/TL2/TL3, (g) TL8/TL9/TL12, (h) TL8/TL9/TL10 and (i) L1/L2/L3, alongside sequencing ladders of the corresponding segments. Samples were as in Fig. 6. Amount of input DNA for uninduced cells in part (b) was 50% more than for other samples analysed in parallel. Note that the 5´ end at np 3211 does not increase uniformly during the time-course of mTERF over-expression, a curious but consistent anomaly (see also Fig. 6a) for which do not have any explanation. The minor bands, which differ in prominence between the genomic regions analysed, are unlikely to be due to nonspecific DNA nicking or polymerase stalling since there is no obvious reason why this would affect some portions of the mitochondrial genome more than others. It may be that they represent genuine DNA ends on the nascent lagging strand.

Supplementary Figure 6

Proposed minimal mTERF binding sites in the 16S/tRNA$^{Leu(UUR)}$/ND1 boundary region. The canonical binding site (highlighted in orange) is the only one in which the pairs of GG residues are on the top (L-) strand. This site is flanked on each side by pairs of binding sites in the opposite orientation (highlighted in yellow). The tRNA$^{Leu(UUR)}$ gene sequence is boxed. Numbering as Ref. 82. The gap represents the original nt 3107, which was later deleted from the sequence upon revision, the gap having been inserted to preserve the original numbering.

FOOTNOTE

The authors' contributions were as follows: AKH, JLOP, AR, SJW, TY, PJK and JNS performed the experimental work: JNS prepared cDNA, SJW recloned mTERF for inducible expression and established inducible cell-lines, JLOP extracted mtDNA and carried out 2DNAGE, partly together with AKH, AR performed LM-PCR, AKH carried out molecular cloning, transfections including RNAi, immunocytochemistry, Western blotting, Q-PCR for copy number determination, EMSA, SELEX and mIP, PJK prepared tissue samples from forensic autopsy and TY performed the initial 2DNAGE analyses to map pause sites. HTJ, IJH and JNS co-supervised the experimental work. HTJ drafted the figures and manuscript.

SUPPLEMENTARY TABLES

SUPPLEMENTARY TABLE 1: EMSA[a] and hybridization probes[b]

Name of probe	Coordinates	Length (bp)	Use	Genes or sequence features covered[a]
TP	15829-15978	150	EMSA	Cyt *b*, $tRNA^{Thr}$, $tRNA^{Pro}$
NCR1	15959-16108	150	EMSA	NCR, $tRNA^{Pro}$, NCR, ETAS1
NCR2	16089-16238	150	EMSA	NCR, ETAS1, TAS, mt5 element
NCR3	16219-16368	150	EMSA	NCR, ETAS2
NCR4	16349-16498	150	EMSA	NCR
NCR5	16479-58	150	EMSA	NCR
NCR	35-611	576	Hybridization	NCR,
OH1	41-190	150	EMSA	NCR, O_H, CSB1, CSB2, CSB3, P_L, P_{H1}, P_{H2} , 12 S rRNA
OH2	171-310	140	EMSA	NCR, O_H, CSB1, TFAM binding sites
OH3	291-440	150	EMSA	NCR, CSB2, CSB3, P_L
OH4	421-570	150	EMSA	NCR, P_L, TFAM binding sites
OH5	520-669	150	EMSA	NCR, TFAM binding sites, P_{H1}, P_{H2} 12S rRNA
Leu	3193-3342	150	EMSA	16S rRNA, $tRNA^{Leu(UUR)}$, ND1
Leu-short	3230-3256	27	EMSA (ds oligo)	$tRNA^{Leu(UUR)}$
ND1.4	3312-3490	179	EMSA	ND1
ND1.5	3451-3600	150	EMSA	ND1
ND1.6	3581-3730	150	EMSA	ND1
ND1	3601-4080	481	Hybridization	ND1
ND1.7	3711-3860	150	EMSA	ND1
ND1.1	3800-3950	150	EMSA	ND1
ND1.2	3931-4080	150	EMSA	ND1
ND1.11	3800-3839	40	EMSA	ND1
ND1.12	3800-3873	74	EMSA	ND1
ND1.13	3813-3893	81	EMSA	ND1
ND1.14	3813-3907	95	EMSA	ND1
ND1.15	3813-3927	115	EMSA	ND1
ND1.16	3847-3950	104	EMSA	ND1
ND1.3	4061-4210	150	EMSA	ND1

IQM1	4201-4350	150	EMSA	ND1, $tRNA^{Ile}$, $tRNA^{Gln}$
IQM2	4331-4480	150	EMSA	$tRNA^{Ile}$, $tRNA^{Gln}$, $tRNA^{Met}$, ND2
ND2	4480-4988	589	Hybridization	ND2
ND2.1	5161-5310	150	EMSA	ND2
ND2.2	5221-5370	150	EMSA	ND2
OL	5685-5834	150	EMSA	$tRNA^{Asn}$, O_L, $tRNA^{Cys}$, $tRNA^{Tyr}$
COI	5831-6244	414	Hybridization	$tRNA^{Tyr}$, COI
A8A6	8475-8625	150	EMSA	A8, A6
A6.1	8501-8650	150	EMSA	A6
A6.2	8631-8780	150	EMSA	A6
A6.3	8761-8910	150	EMSA	A6
A6.4	8891-9040	150	EMSA	A6
A6.5	9021-9170	150	EMSA	A6
A6.6	9151-9300	150	EMSA	A6
ND3.1	9961-10110	150	EMSA	ND3
ND3.2	10091-10240	150	EMSA	ND3
ND3 3	10221-10370	150	EMSA	ND3
ND3.4.	10351-10500	150	EMSA	ND3
ND3.5	10481-10630	150	EMSA	ND3
ND4	11161-11640	480	Hybridization	ND4
ND5-6	14134-14160	27	EMSA (ds oligo)	ND5-ND6 gene junction

[a]Leu-short and ND5-6 were dsDNA oligonucleotides (see text). All other EMSA probes were created by PCR using two 20 nt oligonucleotide primers commencing at the coordinates indicated (82), except for the H-strand primers for ND1.5, ND3.5, A6.1 and IQM1 (17 nt), NCR3, A6.3, A6.4, A6.6 and A8A6 (18 nt), ND3.1, Leu and OL (19 nt), ND3.2 (26 nt) and ND3.4 (27 nt), the L-strand primers for ND1.6 (16 nt), NCR4, A6.5 and OH5 (18 nt), ND3.3 (24 nt) and ND3.5 (27 nt), and both primers for probes ND1.11-16 (all 27 nt).

[b]Hybridization probes were synthesized by PCR using two 20 nt oligonucleotide primers commencing at the coordinates indicated (82).

SUPPLEMENTARY TABLE 2: Oligonucleotides used in LM-PCR

Name of oligonucleotide	Oligonucleotide sequence (5´ to 3´)	Coordinates
TL1	TTAGCGCTGTGATGAGTGTG	4536-4517
TL2	AGTGTGCCTGCAAAGATGGTAG	4522-4501
TL3	TGCTAGGGTGAGTGGTAGGAAGTTTTT	4203-4177
TL4	CGGCTATGAAGAATAGGGCGAAG	3985-3963
TL5	AGTGTGCCTGCAAAGATGGTAG	3812-3788
TL6	AAATAGGAGGCCTAGGTTGAGGTTGA	3618-3593
TL7	GGGCCTTTGCGTAGTTGTATATAGCC	3416-3391
TL8	TCATGTTAGCTTGTTTCAGGTG	5186-5165
TL9	GCTTGTTTCAGGTGCGAGAT	5178-5159
TL10	GGGGTGCCTTGGGTAACCTC	4837-4818
TL11	TGGCAGCTTCTGTGGAACGA	4638-4619
TL12	GGGTATGGGCCCGATAGCTTATT	4438-4416
TL18	GGGTTGATTGCTGTACTTGC	16223-16204
TL19	AGGGGGTTTTGATGTGGATT	16189-16170
TL21	ATGAGGATGGATAGTAATAGGGCAAGG	15645-15619
L1	TAGAGCTGTGCCTAGGACTC	6002-5983
L2	CAGCTCATGCGCCGAATAATAGG	5982-5960
L3	GGTATAGTGTTCCAATGTCTTTGTGGTTTGTAG	5961-5929
H1	CCTCCTAGGCGACCCA	15490-15505
H2	AGACAATTATACCCTAGCCA	15505-15524
H3	CACACGTTCCCCTTAAATAAGACATCACGATG	16537-16569
H5	TCCACCATTAGCACCCAAAGCTAAGATTCT	15976-16005

[a]Coordinates as in Ref. 82.

SUPPLEMENTARY TABLE 3

SELEX analysis of the mTERF binding site and possible binding sites in mtDNA

Position	1	2	3	4	5	6	7	8	9	10	11	12	13	14
SELEX Consensus[a]	t	G	G	t	a	r	a	r	g	T	Y	G	G	t
Actual[b]														
Leu[c]	T	G	G	C	A	G	A	G	C	c	C	G	G	T
ND1.12[d]	a	G	G	G	T	C	A	T	G	a	T	G	G	c
ND1.14[e]	G	G	G	G	T	T	C	G	G	T	T	G	G	T
OH1[f]	a	G	G	A	T	G	A	G	G	c	a	G	G	A

[a]See Table 1 of main paper

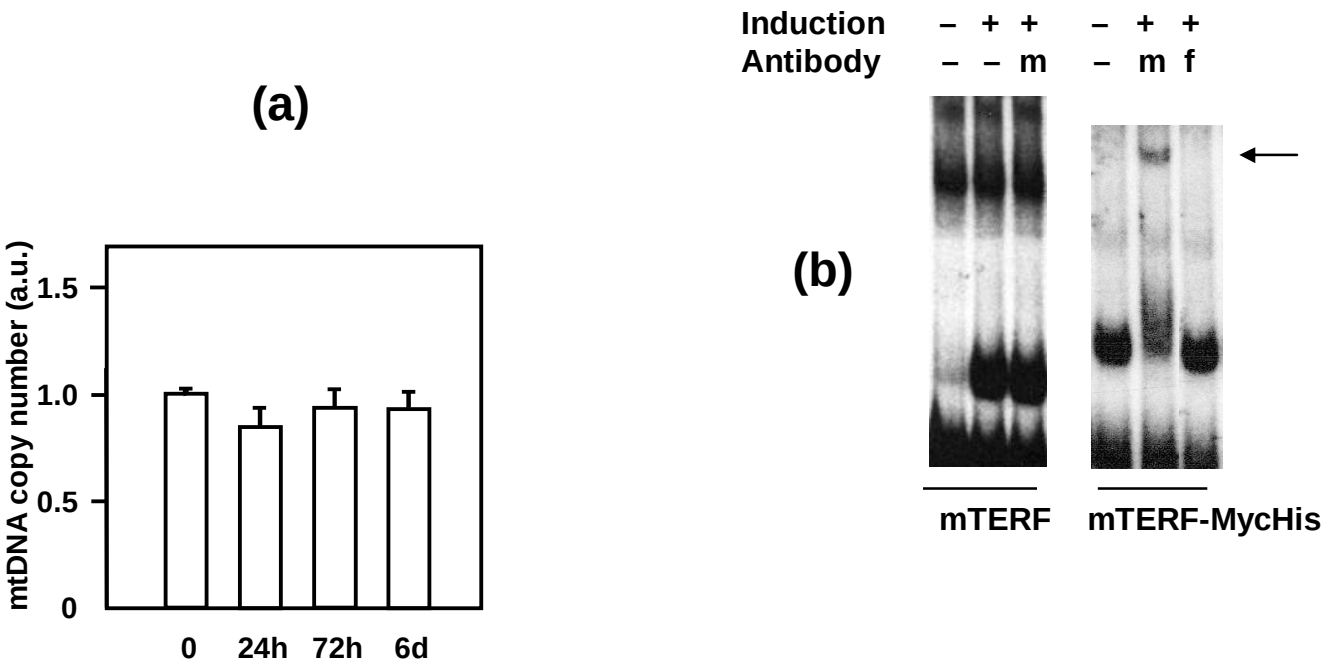
[b]Best matches to consensus within several of the inferred binding-site regions of human mtDNA, based on EMSA

[c]Canonical binding site for mTERF within $tRNA^{Leu(UUR)}$ gene

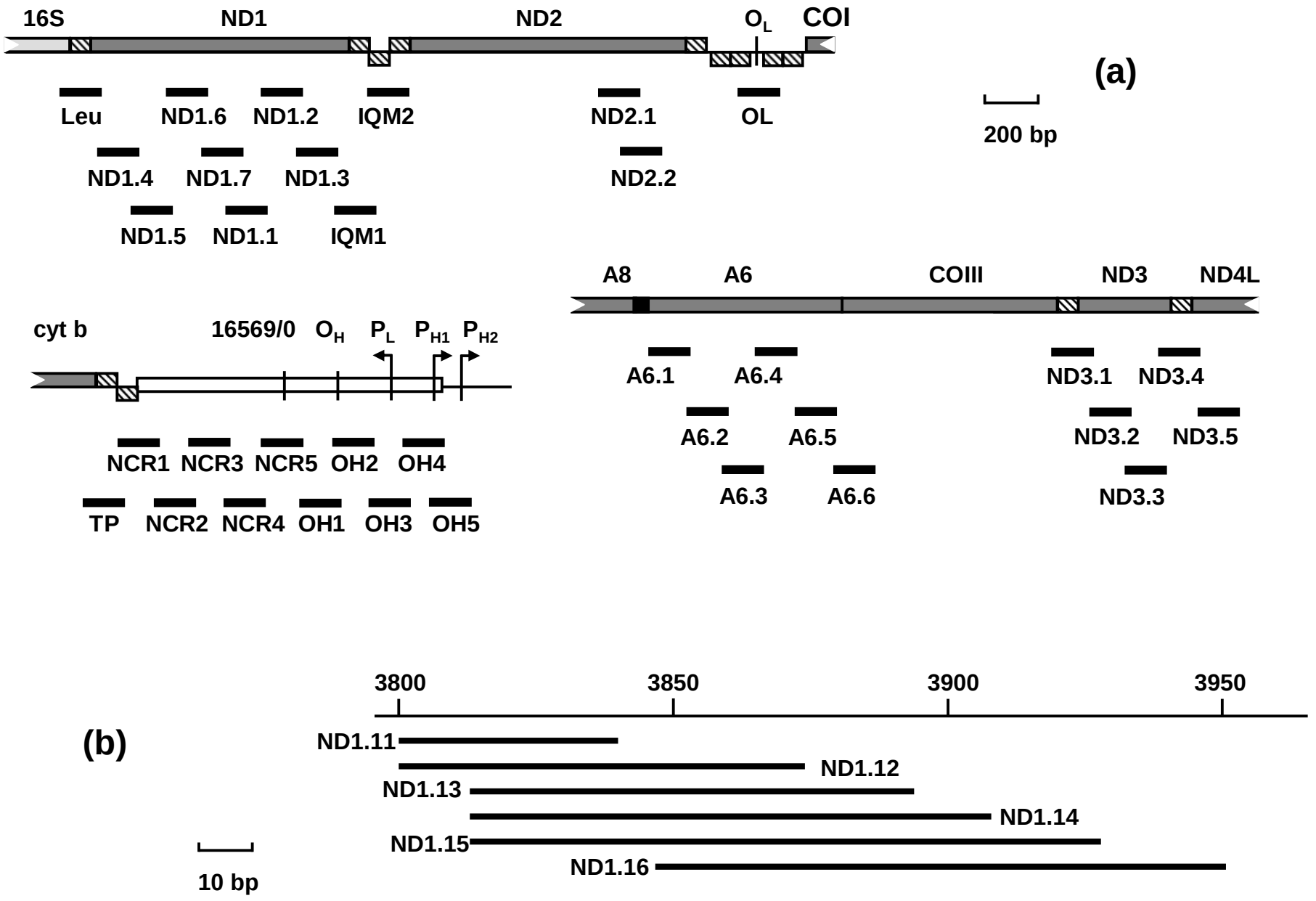
[d]Proposed binding site in ND1 gene between np 3830-3855; see Supplementary Fig. 2d.

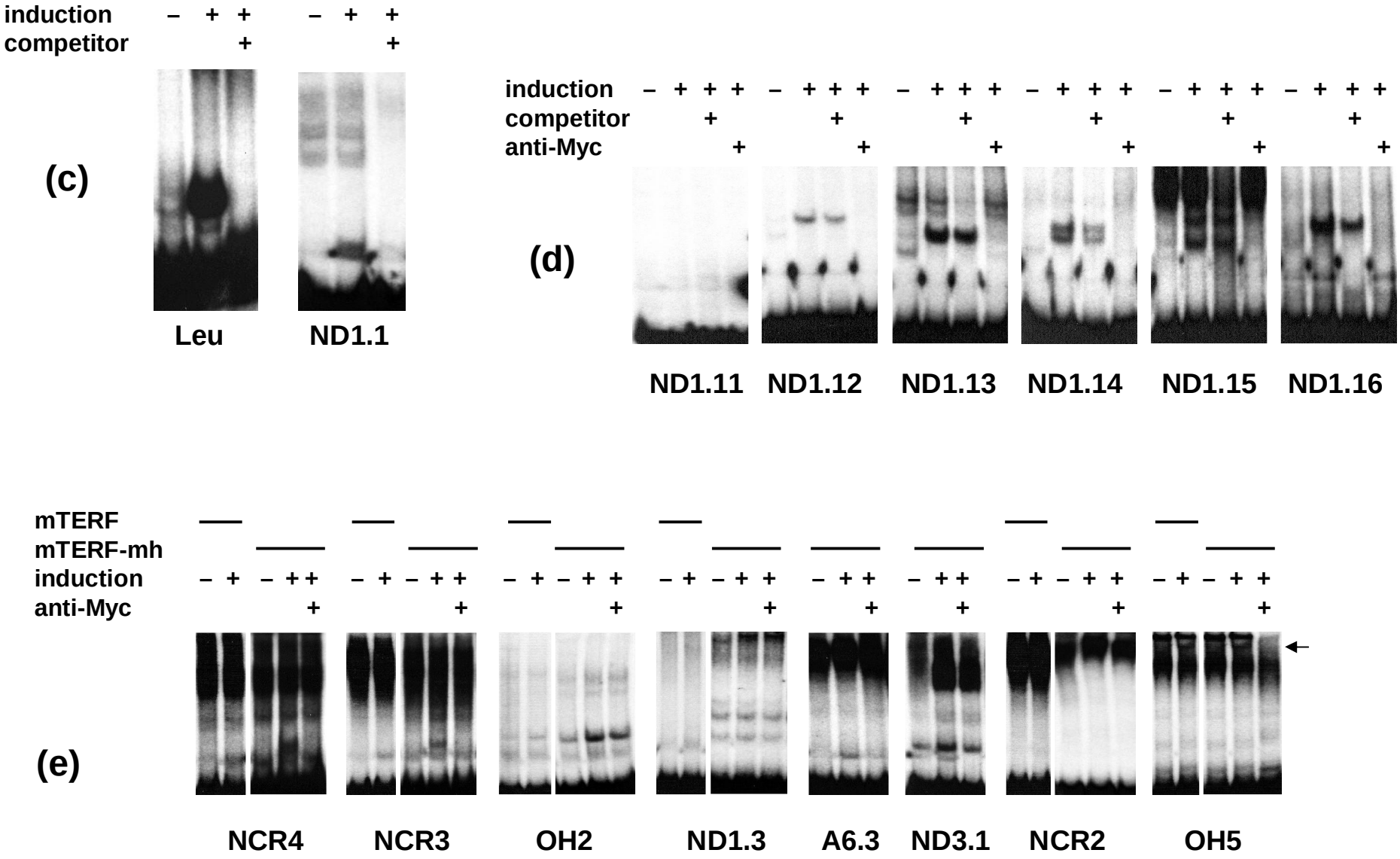
[e]Proposed binding site in ND1 gene between np 3880-307; see Supplementary Fig. 2d.

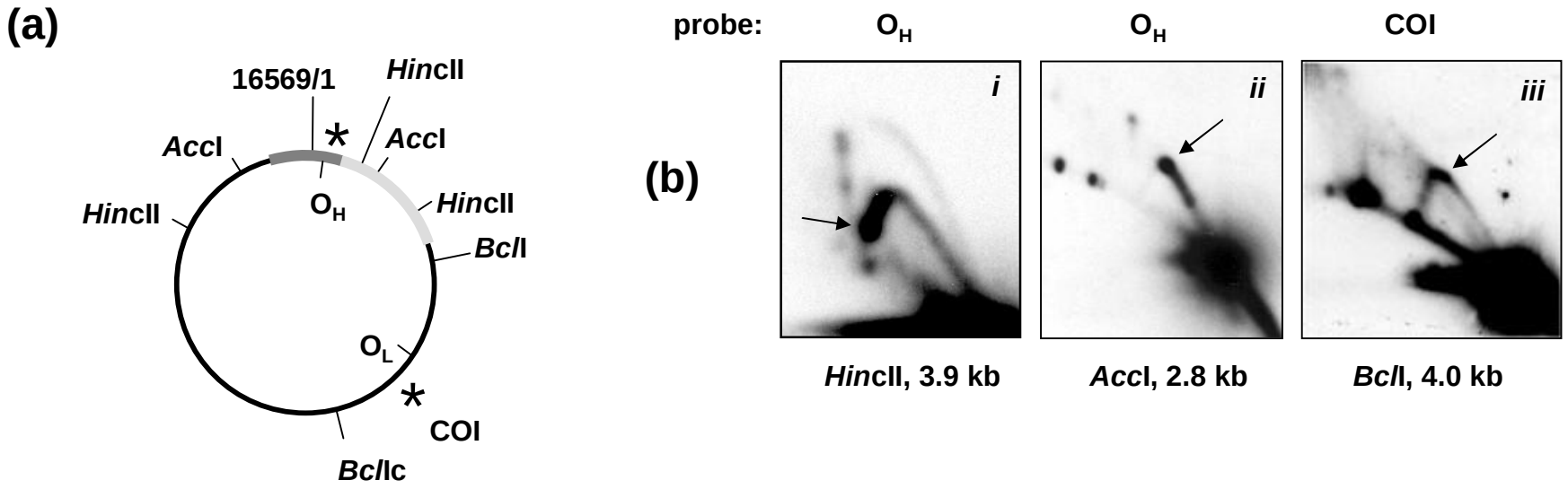
[f]Possible binding site within fragment OH1; see Fig. 2



Hyvärinen et al, Supplementary Figure 1

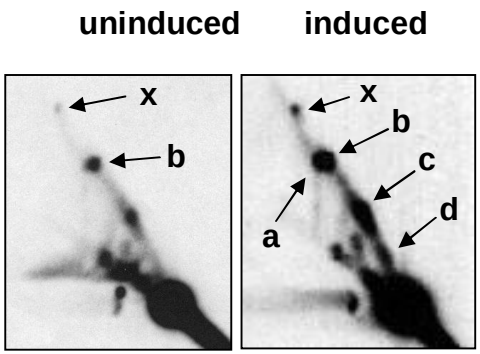

16S
ND1
ND2
O_L
COI
(a)
Leu
ND1.6
ND1.2
IQM2
ND2.1
OL
200 bp
ND1.4
ND1.7
ND1.3
ND2.2
ND1.5
ND1.1
IQM1
A8
A6
COIII
ND3
ND4L
cyt b
16569/0
O_H
P_L
P_{H1}
P_{H2}
A6.1
A6.4
ND3.1
ND3.4
A6.2
A6.5
ND3.2
ND3.5
A6.3
A6.6
ND3.3
NCR1
NCR3
NCR5
OH2
OH4
TP
NCR2
NCR4
OH1
OH3
OH5
(b)
3800
3850
3900
3950
ND1.11
ND1.12
ND1.13
ND1.14
ND1.15
ND1.16
10 bp


induction – + + – + +
competitor + +
(c)
Leu
ND1.1
induction – + + + – + + + – + + + – + + + – + + + – + + +
competitor + + + + + +
anti-Myc + + + + + +
(d)
ND1.11
ND1.12
ND1.13
ND1.14
ND1.15
ND1.16
mTERF
mTERF-mh
induction – + – + + – + – + + – + – + + – + – + + – + + – + + – + – + + – + – + +
anti-Myc + + + + + + + + + + + +
(e)
NCR4
NCR3
OH2
ND1.3
A6.3
ND3.1
NCR2
OH5



Hyvärinen et al, Supplementary Figure 3

(a)



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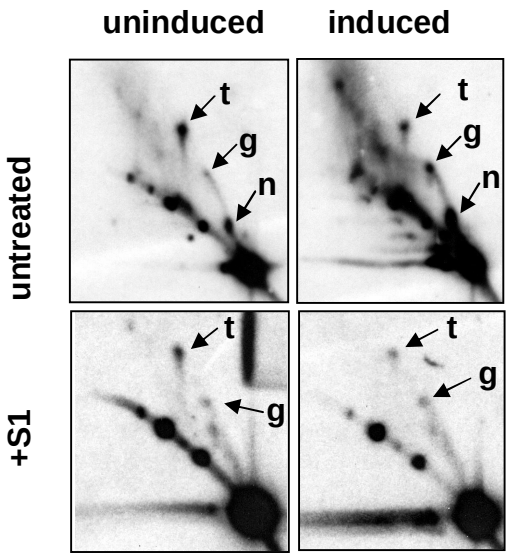
(b)



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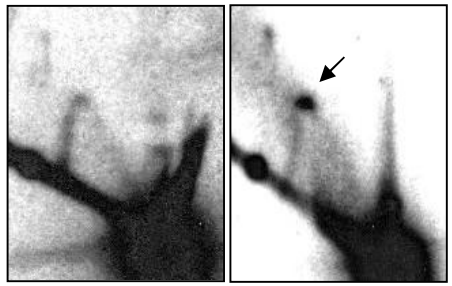
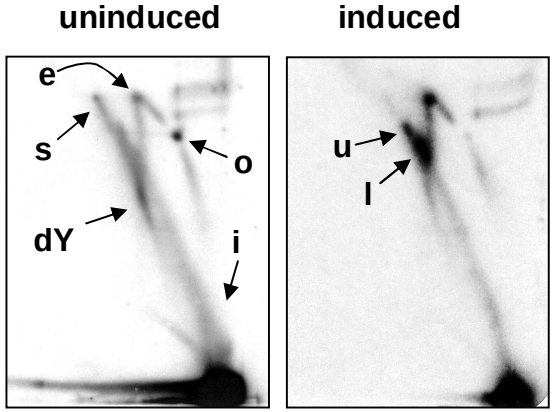
(c)

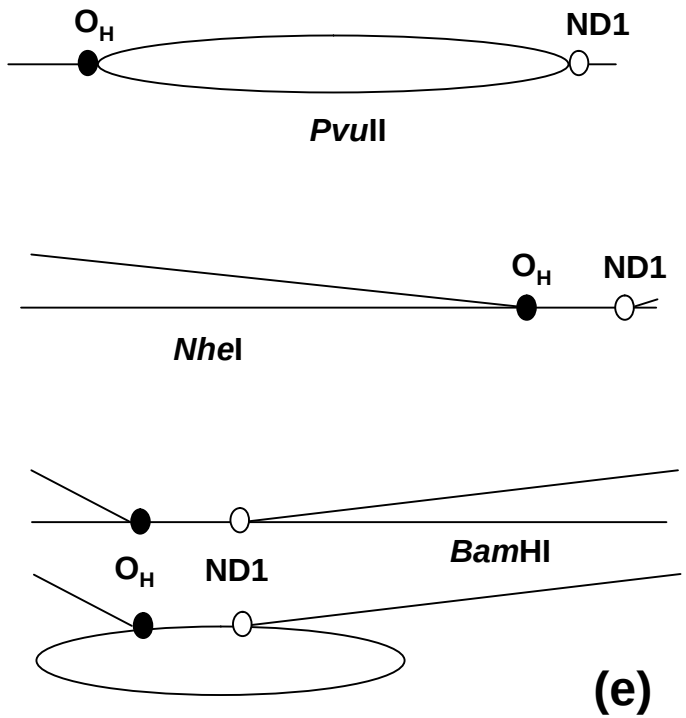


*Bcl*I 3.3 kb, ND4 probe, +S1

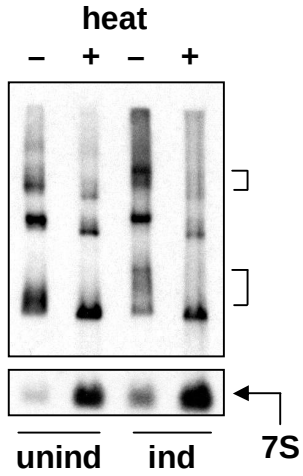
(d)



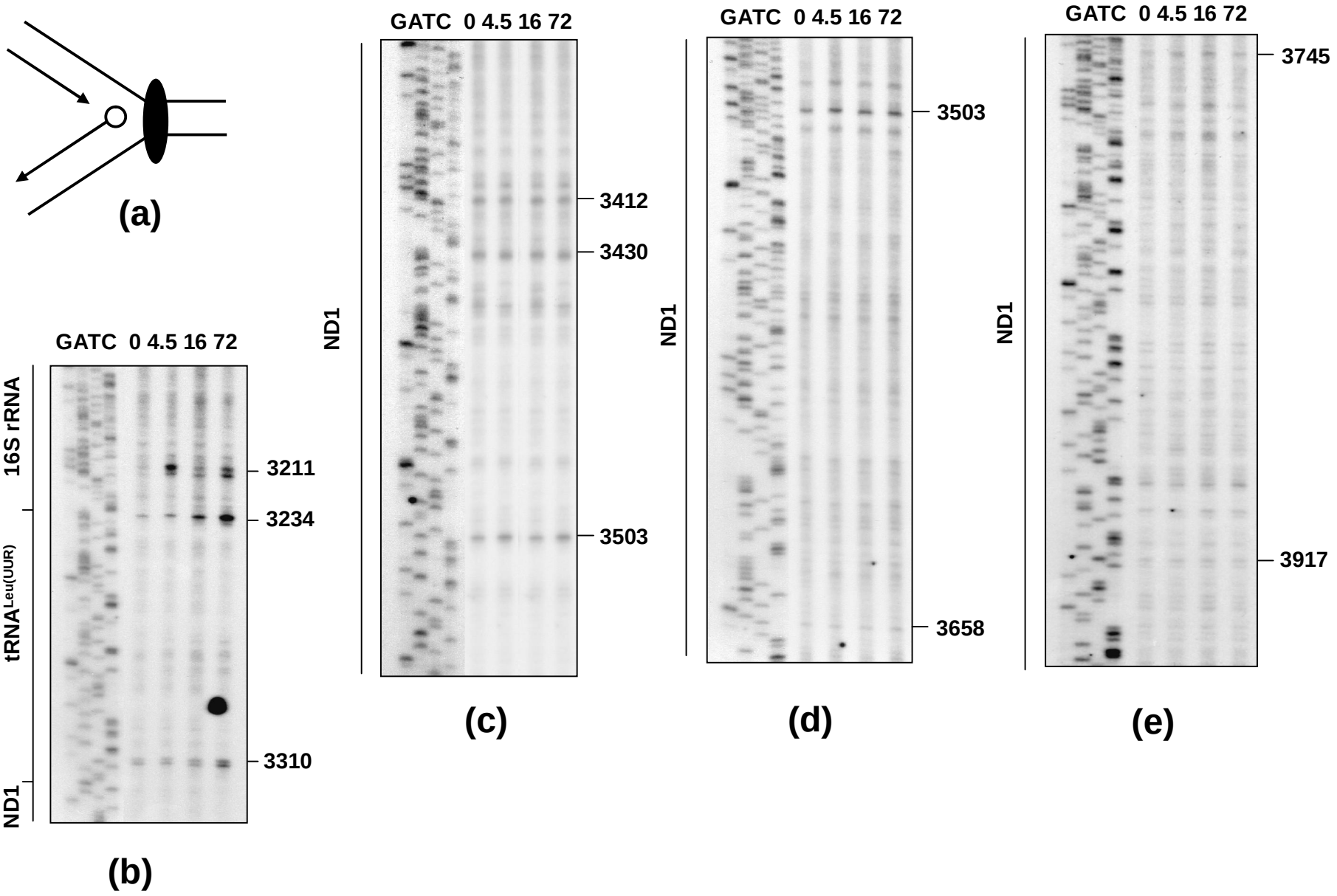
*Bam*HI 16.6 kb, ND2 + ND4 probes

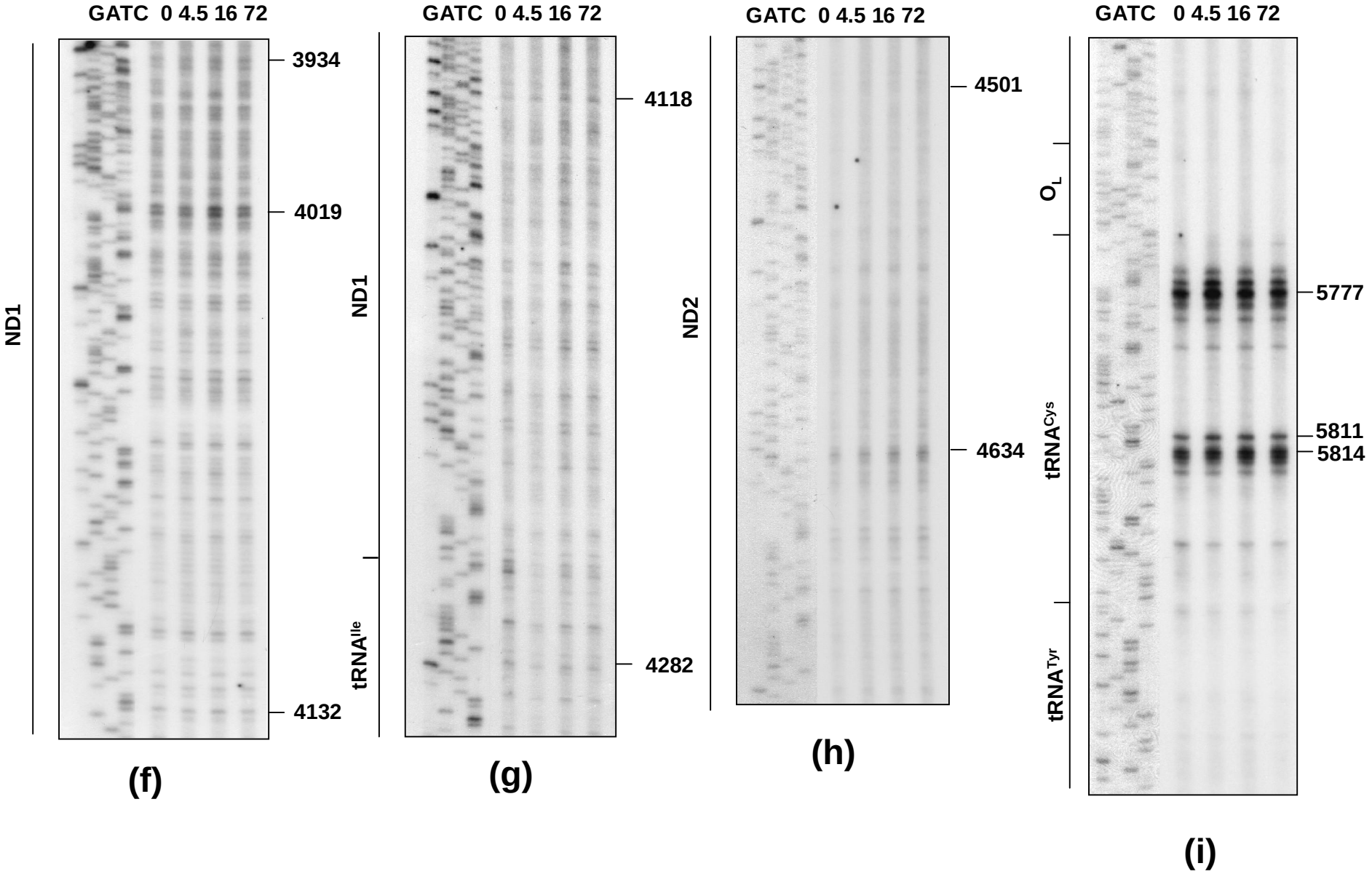


(e)

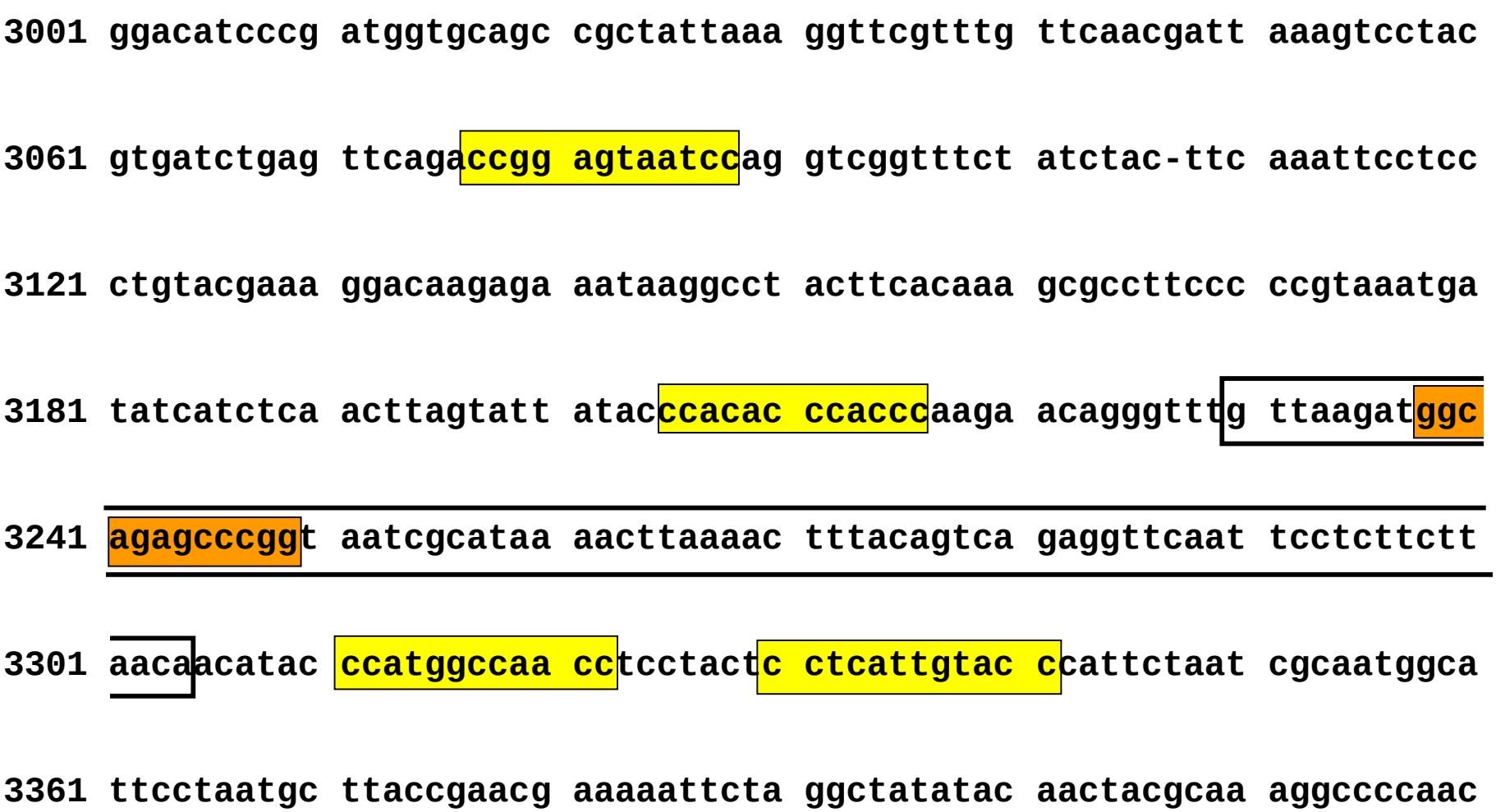


(f)


(a)
GATC 0 4.5 16 72
16S rRNA
tRNALeu(UUR)
ND1
3211
3234
3310
(b)
GATC 0 4.5 16 72
ND1
3412
3430
3503
(c)
GATC 0 4.5 16 72
ND1
3503
3658
(d)
GATC 0 4.5 16 72
ND1
3745
3917
(e)



Hyvärinen et al, Supplementary Figure 5 (page 2 of 2)



Hyvärinen et al, Supplementary Figure 6

BMC Molecular Biology

RESEARCH ARTICLE Open Access

Effects on mitochondrial transcription of manipulating mTERF protein levels in cultured human HEK293 cells

Anne K Hyvärinen[1], Mona K Kumanto[1], Sanna K Marjavaara[1,2], Howard T Jacobs[1*]

Abstract

Background: Based on its activities *in vitro*, the mammalian mitochondrial transcription termination factor mTERF has been proposed to regulate mitochondrial transcription by favouring termination at its high-affinity binding immediately downstream of the rDNA segment of mitochondrial DNA, and initiation selectively at the PH1 site of the heavy-strand promoter. This defines an rDNA transcription unit distinct from the 'global' heavy-strand transcription unit initiating at PH2. However, evidence that the relative activities of the two heavy-strand transcription units are modulated by mTERF *in vivo* is thus far lacking.

Results: To test this hypothesis, we engineered human HEK293-derived cells for over-expression or knockdown of mTERF, and measured the steady-state levels of transcripts belonging to different transcription units, namely $tRNA^{Leu(UUR)}$ and ND1 mRNA for the PH2 transcription unit, and $tRNA^{Phe}$ plus 12S and 16S rRNA for the PH1 transcription unit. The relative levels of 16S rRNA and ND1 mRNA were the same under all conditions tested, although mTERF knockdown resulted in increased levels of transcripts of 12S rRNA. The amount of $tRNA^{Phe}$ relative to $tRNA^{Leu(UUR)}$ was unaffected by mTERF over-expression, altered only slightly by mTERF knockdown, and was unchanged during recovery from ethidium bromide-induced depletion of mitochondrial RNA. mTERF overexpression or knockdown produced a substantial shift (3-5-fold) in the relative abundance of antisense transcripts either side of its high-affinity binding site.

Conclusions: mTERF protein levels materially affect the amount of readthrough transcription on the antisense strand of mtDNA, whilst the effects on sense-strand transcripts are complex, and suggest the influence of compensatory mechanisms.

Background

Mammalian mitochondrial DNA is organized into three multicistronic transcription units (reviewed in [1], Fig. 1A), which give rise to the mature RNAs encoded by the circular genome: two ribosomal RNAs, 22 tRNAs and 11 mRNAs (2 of them bicistronic). Each strand is transcribed in its entirety, employing closely spaced promoters located within the major non-coding region of the genome, namely LSP, the promoter of the light-strand, with a unique initiation site designated PL, and PH1 and PH2, the alternate transcription start sites of the heavy-strand promoter (HSP), which give rise to partially overlapping transcripts. Based on metabolic labeling studies, PH1 and PH2 have been inferred to give rise to distinct primary transcripts of the heavy-strand [2]. PH1 is located within the non-coding region and generates a primary transcript comprising both rRNAs and two tRNAs (-Phe and -Val), terminating at the end of the rDNA region, mainly within the 5' end of the $tRNA^{Leu(UUR)}$ gene [3]. PH2 is located within the coding sequence of $tRNA^{Phe}$ and generates a primary transcript comprising all of the remaining heavy-strand encoded genes. PL generates a primary transcript comprising the entire light strand.

The mechanism by which the transcriptional machinery selects between these different initiation sites, and also effects selective termination at the end of the rDNA, in the case of transcripts initiated at PH1, is

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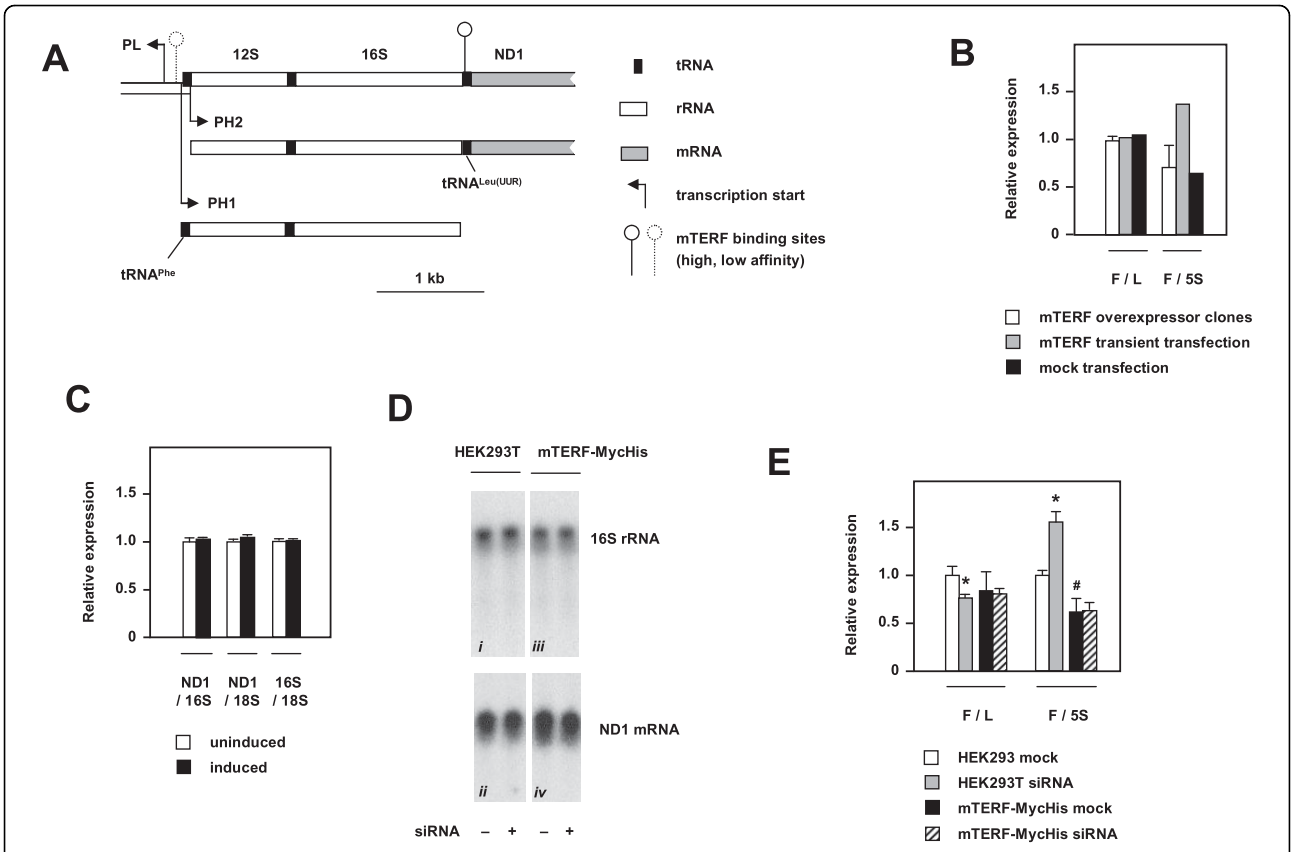

Figure 1 Manipulation of mTERF expression has minimal effects on steady-state levels of mature mitochondrial RNAs. (A) Schematic diagram of the promoter and rDNA region of human mtDNA. Because mTERF binding dictates the use of alternate transcriptional start sites and terminators, $tRNA^{Phe}$ and $tRNA^{Leu(UUR)}$ fall into separate transcription units (PH1 and PH2 respectively). (B) Relative expression of mitochondrial transcripts in cells overexpressing mTERF, based on phosphorimaging of Northern blots probed successively for mitochondrial $tRNA^{Phe}$ and $tRNA^{Leu(UUR)}$ and for 5S rRNA. Data (means ± SD) are signal ratios of $tRNA^{Phe}$ to $tRNA^{Leu(UUR)}$ (F/L) and $tRNA^{Phe}$ to 5S rRNA (F/5S) for the mTERF-overexpressing clones shown in Additional File 1, Fig. S1, normalized to the corresponding ratio in cells stably transfected with empty-vector. Bars shown alongside are based on single reference experiments, using HEK293T cells transiently transfected with the same construct, or mock-transfected. (C) Q-RT-PCR analysis (means ± SD) of mitochondrial transcript levels, plus cytosolic18S rRNA, as indicated, in Flp-In™ T-Rex™-293 cells over-expressing mTERF-MycHis after doxycyclin induction for 3 d (or not induced). Data were normalized, in each case, to the corresponding ratio for uninduced cells. (D) Relative expression of mitochondrial transcripts in cells knocked down for mTERF, as indicated. – denotes mock-transfection. Northern blot probed successively for 16S rRNA and ND1 mRNA, as shown. The panels represent non-adjacent pairs of lanes from the same exposure of the same gel. (E) Relative expression of mitochondrial transcripts in cells knocked down for mTERF, as indicated, calculated from Northern blot data as in (B), normalized to the corresponding ratio in mock-transfected HEK293T cells. * indicates significant differences from the corresponding mock-transfected cells, and # a significant difference between cell-lines (*t*-test, *p* values as in text). For original blots see Additional File 1, Fig. S1C. Note that additional Q-RT-PCR data on levels of 12S rRNA gene transcripts are shown in Fig. 3.

incompletely understood. It can be manipulated *in organello* by various drugs and by ATP [4-6]. The mitochondrial RNA polymerase comprises a single catalytic subunit, MTRPOL, plus an accessory factor, TFB2M, required for formation of the initiation complex *in vitro* at both HSP and LSP [7,8], together with mitochondrial transcription factor A (TFAM), which is needed for promoter-dependent transcription *in vitro* [7,9]. TFAM has a natural binding affinity for DNA and has been suggested also to play a more general role in organizing the mitochondrial chromosome, analogous with bacterial HU or eukaryotic and archaeal histones. A third factor, mTERF, with sequence-specific binding affinity for a sequence located within the $tRNA^{Leu(UUR)}$ gene immediately downstream of the rDNA [10,11], has been proposed to play a key role in both initiation and termination of the PH1 transcription unit [12].

mTERF has selective termination activity *in vitro* on templates containing its high-affinity binding site in the $tRNA^{Leu(UUR)}$ gene [10,13]. In crude extracts [14], as well as in a reconstituted system based on recombinant proteins [15], this activity appears to be bidirectional, but operates in the latter case more efficiently in the reverse direction, i.e. to terminate transcription initiating from the LSP side more efficiently than from HSP [15]. Based on the fact that it has weak binding to other sites

in mtDNA, including the promoter region [12,16,17], it has been proposed that mTERF favours transcription of the PH1 transcription unit by simultaneous binding to the promoter and to the terminator region, creating a loop structure that can be visualized *in vitro* [12]. The level of active mTERF would thus act as a fine tuning of the relative production of rRNA and mRNA.

There are, however, some problems associated with this model. First, efficient transcription from PH1 *in vitro* does not require mTERF (although does appear to be stimulated by it [18]), whereas transcription from PH2 *in vitro* is weak [18]. Second, measurements of the relative half-lives of mitochondrial rRNAs and mRNAs in cultured cells [19] indicate that post-transcriptional regulation is substantial and may in fact be sufficient to maintain the different transcript levels seen *in vivo*, without the need for any differential regulation of transcription from the PH1 and PH2 transcription units. Note that, although the synthesis rates of mitochondrial rRNAs and mRNAs appear to be very different in both cultured cells [19] and rat liver [20], 'synthesis rate' here includes RNA processing as well as transcription. *In organello*, the combined rate of accumulation of pre-rRNA plus mature rRNA is, in fact, lower than that of mRNA [6]. Third, no modulation of transcription from the two initiation sites correlating with mTERF activity has ever been convincingly demonstrated *in vivo*. Fourth, in cells bearing the 3423A > G mutation, which greatly impairs mTERF binding *in vitro*, there is no alteration in the relative levels of 16S rRNA and ND1 mRNA [21,22], and no alteration in site occupancy *in vivo*, based on footprinting studies [21]. Fifth, decreased levels of mTERF expression in *Mpv17* knockout mice are associated with globally increased mitochondrial transcription [23], suggesting rather than mTERF may function *in vivo* as a negative but general regulator of transcription. Finally, whilst recombinant mTERF is active in a reconstituted system *in vitro* [15], its activity in the presence of less pure mitochondrial extracts is subject to post-translational modifications and/or the presence of other proteins [11-13,18,24], raising doubts as to whether and how it influences transcription *in vivo*.

mTERF is a member of a family of organellar proteins proposed to interact with DNA to produce a variety of outcomes [25]. In mammals, two homologues of mTERF, MTERFD1 (mTERF3) and MTERD3 (mTERF2), have been shown to influence mitochondrial RNA levels and have been proposed to act as regulators of transcription from LSP [26,27], with consequent effects on oxidative phosphorylation mediated brought about by altered translation, as seen also in *Drosophila* [28]. However, neither mTERF homologue has been conclusively demonstrated to have high-affinity sequence-specific binding to DNA [26,27,29]. Homologues of mTERF in invertebrates have been demonstrated to influence both RNA and DNA synthesis *in vitro*, but here too, there is only weak evidence for a specific role *in vivo*. The mTERF-homologue in sea urchins, mtDBP, binds to at least two sites in the mitochondrial genome [30] and exhibits bidirectional transcription termination activity *in vitro* in the presence of human mitochondrial RNA polymerase, although it acts unidirectionally in combination with phage polymerases [31]. It also impedes the progress of DNA polymerase bidirectionally, acting as a contrahelicase *in vitro* [32], suggesting a possible role in DNA replication. A role for mTERF in mammalian mtDNA replication is also suggested by the observation that the level of mTERF expression in cultured human cells influences replication pausing in the vicinity of mTERF binding sites [16]. The *Drosophila* mTERF homologue mTTF binds to two putative transcriptional terminators [33], acting *in vitro* with similar directional properties to mtDBP [34]. Manipulation of DmTTF levels in cultured cells leads to effects on transcript levels consistent with it acting in the manner hypothesized for mTERF, i.e. as a regulator of termination (bidirectionally) and also of promoter activity [35].

The difficulty of interpreting *in vitro* experiments, and the open questions regarding the role of mTERF *in vivo*, prompted us to address the issue of whether and how mTERF activity influences mitochondrial RNA levels in cultured human cells. Clearly, if mTERF is a regulator of mitochondrial transcription *in vivo*, via a model as proposed, up- or down-regulation of its expression should influence mitochondrial RNA levels in a predictable fashion. We therefore undertook a study of mitochondrial transcripts in cells over-expressing or knocked down for mTERF. Surprisingly, we found that varying the level of mTERF over a wide range has only a small effect on the levels of sense-strand transcripts of the mitochondrial genome in the rDNA region. Conversely, we detected a clear effect on the relative amounts of antisense transcripts on the two sides of the high-affinity binding site. These findings support a role for mTERF in influencing mitochondrial transcription *in vivo*, but not in setting the levels of mature mitochondrial transcripts.

Results

Over-expression of mTERF does not alter steady-state levels of mature mitochondrial RNAs

To evaluate whether the expression level of mTERF influences the steady-state levels of the mature mitochondrial transcripts encoded on either side of its high-affinity binding site, we generated a series of transfected HEK293T cell clones stably over-expressing the natural mTERF protein. Expression of the mTERF transgene

was verified at the RNA level by Q-RT-PCR (Additional File 1, Fig. S1A) and at the protein level by the substantial increase in DNA-binding capacity at the high-affinity mTERF binding site, as judged by EMSA (electrophoretic mobility shift assay, Additional File 1, Fig. S1B).

We analysed two parameters which we considered diagnostic for the relative utilization of the two heavy-strand transcription units predicted by the classic model of mammalian mtDNA transcription (Fig. 1A). The first is the relative amounts of tRNAs -Phe and -Leu(UUR), which are exclusively produced by transcription from PH1 and PH2, respectively, according to the classical model. The second is the relative amounts of mature 16S rRNA and ND1 mRNA. The latter is synthesized via transcription from PH2, whereas the former has been proposed to be generated mainly or exclusively from transcription initiating at PH1, although it has not been formally excluded that transcription from PH2 also contributes some of the 16S rRNA. We found that the relative amounts of tRNAs -Phe and -Leu(UUR) in different cell-clones over-expressing natural mTERF was indistinguishable from that in control cells transfected with empty vector (Fig. 1B), and was also unchanged in cells transiently transfected with the mTERF overexpression construct (or mock transfected cells). The global amount of mitochondrial transcription, as measured by the ratio of $tRNA^{Phe}$ to cytosolic 5S rRNA was more variable, but showed no systematic relation to mTERF overexpression (Fig. 1B). We also found no detectable alteration in the relative amounts of mature NDI1 mRNA and 16S rRNA between mTERF over-expressing clones and control cells, based on Northern blots (Fig. 1D: compare lanes 1 of panels *i* and *ii* [control cells] with lanes 1 of panels *iii* and *iv* [over-expressing cells]). In an effort to quantify any such effect and avoid possible influences of cell background, we also used Q-RT-PCR to analyse transcripts of the 16S and ND1 genes in RNA extracted from Flp-In™ T-Rex™-293 cells stably transfected with the mTERF-MycHis construct, in which expression of mTERF can be induced by doxycycline (Fig. 1C). We found no differences in the relative amounts of transcripts from these two genes, nor in the ratio of either to cytosolic 18S rRNA transcripts.

Effects of mTERF knockdown on steady-state levels of mature mitochondrial RNAs

In previous studies [16] we noted that transfection with an siRNA directed against mTERF suppressed most of the binding activity at the high-affinity mTERF binding site, as judged by EMSA [16]. We therefore compared the relative levels of mitochondrial transcripts in cells knocked down for mTERF. Northern blots probed successively for 16S rRNA and ND1 mRNA showed no difference in the relative levels of these mature transcripts in HEK293T cells after prolonged treatment (7 d) with an mTERF-specific siRNA (Fig. 1D: compare lanes 1 and 2 of panels *i* and *ii*), nor in mTERF-MycHis overexpressing cells knocked down for mTERF (Fig. 1D: compares lanes 1 and 2 of panels *iii* and *iv*). mTERF knockdown in HEK293T cells did, however, produce a small but significant decrease in the relative amount of $tRNA^{Phe}$ compared with $tRNA^{Leu(UUR)}$, (Fig. 1E, *t*-test, $p < 0.05$), accompanied by an increase in the overall amount of mitochondrial tRNAs, represented by the ratio of mitochondrial $tRNA^{Phe}$ to cytosolic 5S rRNA (*t*-test, $p < 0.01$). siRNA treatment of mTERF-MycHis overexpressing cells caused no significant alteration in mitochondrial tRNAs (Fig. 1E), compared with mock-transfected cells. Note also that cells overexpressing mTERF-MycHis showed no clear difference from HEK293T cells in the relative levels of mitochondrial $tRNA^{Phe}$ and $tRNA^{Leu(UUR)}$ (Fig. 1E), although mitochondrial tRNA levels globally were lower than in untransfected HEK293T cells (*t*-test, $p < 0.01$).

Manipulation of mTERF expression does not alter the relative levels of mitochondrial tRNAs during recovery from mitochondrial RNA depletion

Reasoning that the steady-state levels of mature mitochondrial transcripts may not accurately reflect their transcription rates *in vivo*, due to the influence of post-transcriptional processing, we set out to study whether the level of mTERF expression can influence the re-accumulation of tRNA transcripts belonging to the PH1 and PH2 transcription units during recovery from ethidium bromide (EtBr)-induced depletion of mitochondrial RNA. We compared the ratio of mitochondrial tRNAs -Phe and -Leu(UUR) in stably transfected cells overexpressing mTERF-with that in empty vector-transfected cells over 2 days of EtBr treatment followed by 5 days of recovery (Fig. 2A, Additional File 1, Fig. S2A). In both cell lines the ratio fell substantially during depletion, reflecting the much shorter half-life of $tRNA^{Phe}$, but then recovered to levels higher than those seen in untreated cells, before decreasing again gradually, towards the starting value. This may indicate that the PH2 transcription unit is used preferentially during recovery from depletion. However, this did not appear to be influenced by the level of mTERF, since the same pattern was seen in control cells and in three separately analysed overexpressor cell lines, as well as in cells knocked down for mTERF by treatment with the mTERF-specific siRNA, which behaved indistinguishably from mock-transfected cells (Fig. 2B). The overall kinetics of recovery of mitochondrial transcripts compared with cytosolic 5S rRNA was also similar, comparing cells over-expressing mTERF with control cells (Additional File 1, Fig. S2B), and comparing cells

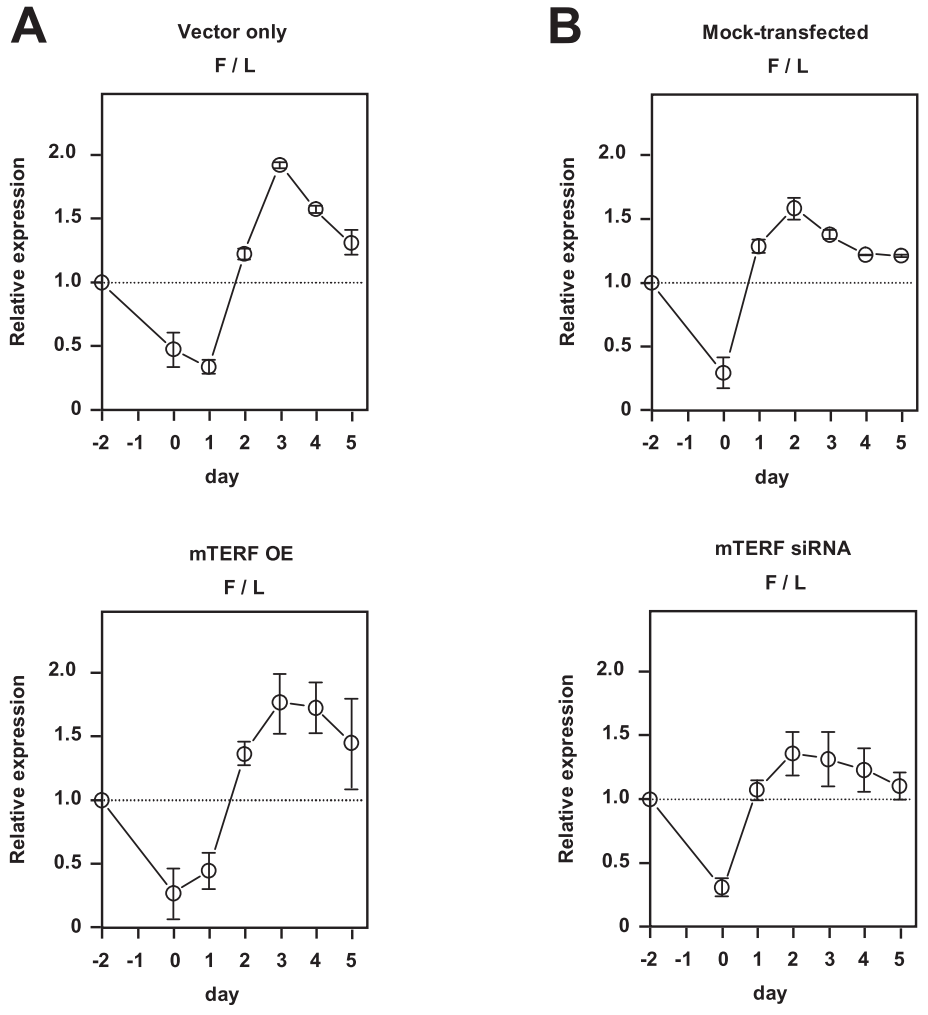

Figure 2 Manipulation of mTERF expression has minimal effects on levels of mature mitochondrial RNAs during recovery from EtBr-induced depletion. Relative expression of mitochondrial transcripts in cells overexpressing mTERF, based on phosphorimaging of Northern blots probed successively for mitochondrial $tRNA^{Phe}$ and $tRNA^{Leu(UUR)}$. Data (means ± SD) are ratios of $tRNA^{Phe}$ to $tRNA^{Leu(UUR)}$ (F/L) normalized to the ratio at the start of the experiment (time-point –2 d). (A) Cells stably transfected with empty-vector (as shown in Additional File 1, Fig. S1B) or mTERF overexpression (OE) construct (clone 3, as shown in Additional File 1, Fig. S1). Overexpressor clones 1 and 2 gave similar results: sample blots shown in Additional File 1, Fig. S2A. (B) Cells treated with mTERF-specific siRNA (or mock-transfected) prior to the addition of EtBr (day –2) and again 2 days after removal of EtBr (day 2). Days 1-5 indicate the period of subsequent recovery. For equivalent data on ratio of $tRNA^{Phe}$ to 5S rRNA from the same experiment see Additional File 1, Fig. S2B, C.

knocked down by mTERF-specific siRNA with mock-transfected cells (Additional File 1, Fig. S2C).

Manipulation of mTERF expression influences both sense- and antisense-strand transcription

Since the effects of manipulating mTERF expression on the levels of mature 16S rRNA and ND1 mRNA did not reveal any significant changes, we used strand-specific quantitative RT-PCR to analyse effects on the levels of both sense-strand and antisense-strand transcripts derived from specific regions of these genes either side of the high-affinity mTERF binding site. We analyzed the relative levels of antisense transcripts from portions of the ND1 and 16S genes in three contexts in which mTERF expression was manipulated (Fig. 3B). In cell clones stably overexpressing mTERF the relative level of anti-16S to anti-ND1 RNA was decreased compared to control cells transfected with the empty vector, although this difference was only statistically significant in one of the two clones studied. Induction of mTERF expression in Flp-In™ T-Rex™-293 cells stably transfected with the mTERF construct also resulted in a substantial and statistically robust decrease in the anti-16S:anti-ND1 ratio, whereas transfection of HEK293T cells with an shRNA targeted on mTERF resulted in the opposite effect, i.e., a significant

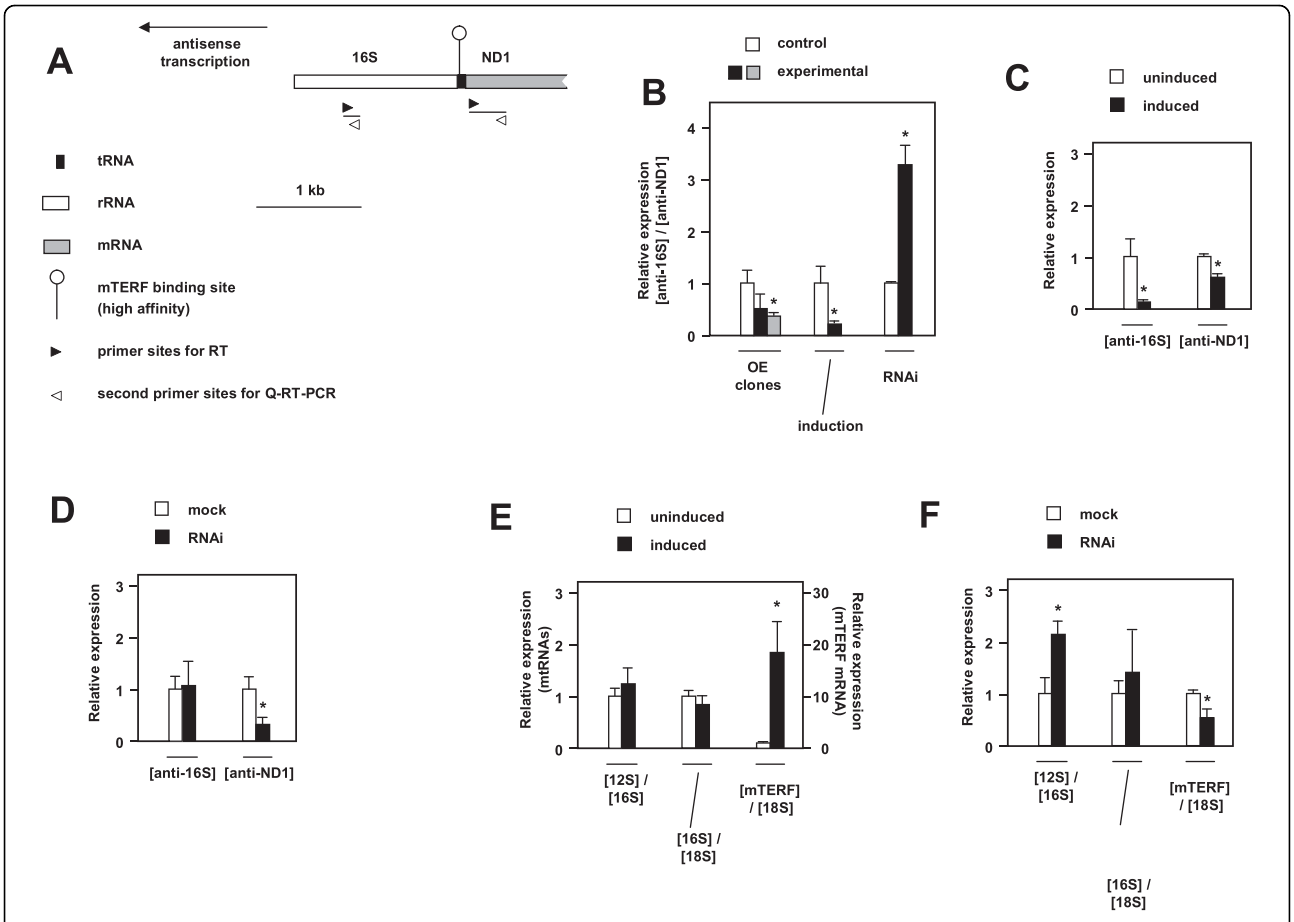

Figure 3 Manipulation of mTERF expression affects relative levels of antisense transcripts of the 16S rRNA and ND1 genes. (A) Schematic diagram of 16S rDNA-ND1 region of human mtDNA. For full details of primer sequences and location, see Additional File 1, Table S1. (B) Relative steady-state levels of anti-16S and anti-ND1 transcripts, determined by Q-RT-PCR using proximity probe hybridization (probe sets R1, N1 and C1 for normalization), after various manipulations of mTERF expression, i.e. two mTERF overexpressor clones (OE) compared with vector-transfected cells, doxycyclin-induced *versus* uninduced Flp-In™ T-Rex™-293 cells transfected with mTERF expression construct, and HEK293T cells transfected with mTERF-targeted shRNA *versus* mock-transfected cells. In each case, data were normalized to the corresponding control cells. * denotes statistically significant differences from control cells (*t* test, $p < 0.02$). (C, D) Relative changes in anti-16S and anti-ND1 transcripts, based on replotting of data from the experiment of panel B for each transcript individually, (C) following induced expression of mTERF in Flp-In™ T-Rex™-293 cells and (D) in HEK293T cells transfected with mTERF-targeted shRNA *versus* mock-transfected cells. Data were normalized to values for corresponding untreated control cells, using 18S as internal normalization standard. * denotes statistically significant differences from corresponding control cells (*t* test, $p < 0.01$). (E, F) Relative steady-state levels of 12S, 16S and 18S sense-strand transcripts, as determined by Q-RT-PCR using of proximity probe hybridization (primer sets T1, R2, C1 respectively, as described in Additional File 1, Table S1), and of mTERF mRNA relative to 18S rRNA (probes sets M1 and C1, see Additional File 1, Table S1), (E) following induced expression of mTERF in Flp-In™ T-Rex™-293 cells and (F) in HEK293T cells transfected with mTERF-targeted shRNA versus mock-transfected cells. * denotes statistically significant differences (*t* test, $p < 0.02$). See also Additional File 1, Fig. S3.

large increase in the relative amount of anti-16S RNA. Notably, the pattern of changes in each RNA differed in the two cases (over-expression and knockdown), when comparing its level in treated versus untreated cells (Fig. 3C, D). Induced overexpression, which resulted in a 20-fold increase in mTERF mRNA (Fig. 3E), produced a severe decrease in antisense transcripts from the 16S gene, but also a small decrease in the level of anti-ND1 (Fig. 3C). mTERF knockdown (by a factor of 2 at the RNA level: Fig. 3F) produced no significant effect on antisense transcripts of 16S, but a sharp drop in the level of anti-ND1 (Fig. 3D).

We validated the main findings using a second primer set (Additional File 1, Fig. S3B), which was also used to test effects on the relative amounts of sense-strand transcripts from the 16S and ND1 genes, which were found to be unaffected by these manipulations, as expected from the analysis of mature transcripts by Northern blots (Fig. 1). In addition, we analysed effects on sense-strand transcript from the 12S rRNA gene, and

determined the levels of sense-strand transcripts of both mitochondrial rRNAs relative to cytosolic 18S rRNA (Fig. 3E, F). Under conditions of induced over-expression of mTERF, sense-strand transcripts of 12S rRNA and of 16S rRNA were unchanged relative to each other and to cytosolic 18S rRNA (Fig. 3E). However, we did detect a significant *increase* in sense-strand 12S rRNA transcripts in cells knocked down for mTERF (Fig. 3F).

Discussion

mTERF and heavy-strand promoter modulation

In this study we investigated the effects of manipulating the expression level of mTERF on the relative levels of different mitochondrial transcripts. Under all conditions tested we failed to detect any significant effects on the relative levels of mature 16S rRNA and ND1 mRNA (Fig. 1C, 1D, Additional File 1, S3C). Over-expression of a tagged mTERF variant, which resulted in the greatest increase in DNA-binding activity that we were able to generate (Fig. 5C of [16]), produced no significant change in the relative levels of the mitochondrial tRNAs tested, with only a minor decrease in their overall abundance (Fig. 1E). Induced 20-fold over-expression of natural mTERF in a controlled nuclear background also did not alter the ratio of mature 16S rRNA to ND1 mRNA, nor were the levels of 16S or ND1 transcripts affected relative to transcripts of cytosolic 18S rRNA or 12S rRNA (Fig. 1C, 3E). Knockdown of mTERF resulted in a very modest decrease in the level of $tRNA^{Phe}$ relative to $tRNA^{Leu(UUR)}$. However, this was not sufficient to generate any significant change in the kinetics of recovery of mitochondrial tRNA levels following EtBr-induced depletion.

We did, however, obtain two piece of evidence that mTERF knockdown is not inert as regards transcription of the mitochondrial heavy strand. Firstly, we observed, by Northern blots, a small increase in the amount of mitochondrial tRNAs belonging to each of the heavy-strand transcription units, relative to cytosolic 5S rRNA (Fig. 1E) in normal cells after mTERF knockdown. Secondly, the level of sense-strand 12S rRNA gene transcripts analysed by quantitative RT-PCR was significantly increased relative to sense-strand 16S rRNA or cytosolic 18S rRNA gene transcripts (Fig. 3F), in normal cells knocked down for mTERF. However, the levels of sense-strand 16S and ND1 transcripts relative to each other or to 18S were not significantly affected (Fig. 3F). This suggests the existence of a compensatory mechanism, whereby decreased mTERF levels, which might otherwise impair 16S rRNA biogenesis, generate a signal for globally increased mitochondrial transcription (or decreased turnover) to overcome any such defect. It may also be noted that the effects of knockdown may be underestimated due to the rather limited decrease in mTERF mRNA level that we were able to achieve in these experiments. A 50% decrease is not untypical in cultured mammalian cells in cases where knockdown of a given gene may provoke a growth defect, even just a transient one, compared with untransfected cells in the culture. Thus, the effects we observed may likely represent a combination of normal expression in almost half the cells, plus greatly reduced expression in the remaining cells.

Nevertheless, our findings imply that the expression level of mTERF does not determine, in a simple manner, the relative steady-state levels of transcripts belonging to the two transcription units of the heavy-stand. Although mTERF was previously shown to stimulate transcription *in vitro* from PH1 in a comparatively crude system [12,18], it may be noted that no such effect was seen when purified, recombinant proteins were used [15], or even in crude extracts using DNA-affinity purified mTERF [18].

Our results indicate that even if mTERF levels do influence transcriptional readthrough, a compensatory response nevertheless adjusts the relative output of different transcripts belonging to the two heavy-strand transcription units. This may involve the modulation of transcriptional initiation, post-transcriptional processing or RNA turnover. Our findings are consistent with previous reports of the action of thyroid hormone [36] or variation in ATP supply [37], both of which can influence the relative rates of transcriptional of initiation at PH1 and PH2 without any effect on that at the high-affinity mTERF binding site. It is also possible that mTERF might have a different physiological function, and that its effects on transcription are accommodated by modulating other components of the mitochondrial RNA synthesis machinery.

Is mTERF activity in HEK293 cells physiological?

All of the current study was conducted in one cell-line and its derivatives which, as a cancer cell-line, may not behave in a physiologically normal manner. We considered the hypothesis that mTERF levels may, in other cell-types, have a more profound effect on mitochondrial transcription but that, in HEK293 cells, mTERF could be present in such excess that neither over-expression nor any amount of knockdown achievable by RNAi technology influences its functional level. However, from available gene expression data (biogps.gnf.org) the range of expression of mTERF in different cell-types *in vivo*, plus primary tumours and cell-lines including HEK293 and its derivatives, is only of the order of 2-5 fold. Furthermore, in HEK293T cells mTERF is expressed at very close to the median level for all cells investigated. Therefore, the range of expression achieved in the present study (~40-fold at the RNA

level, Fig. 3E, F) far exceeds that known to be experienced *in vivo*.

Another possibility, given the wealth of previous data indicating possible post-translational regulation of the transcriptional activity of mTERF, is that mTERF is constitutively inactivated in HEK293T cells, regardless of its expression level. Although we analysed DNA-binding activity as well as RNA levels, some mTERF preparations that are competent for DNA binding are nevertheless unable to influence transcription *in vitro* [11,24]. This is unlikely, however, since the patterns of mitochondrial transcripts in HEK cells, and their responses to other manipulations, such as increases in the level of TFAM [38], are similar to other cultured cells and *in vivo* tissues.

Thiamphenicol treatment, which alters the representation of PH1- and PH2-derived transcripts in a manner similar to thyroid hormone treatment, is able to modify the EMSA signal at the high-affinity mTERF binding site, whilst leaving the actual levels of mTERF polypeptide unaffected [39]. This may indicate that a post-translational modification of mTERF could modulate both its DNA-binding and its transcriptional properties *in vivo*, but is equally consistent with the notion that another factor, capable of binding in this region, is involved.

Final resolution of these issues will require the creation of an *in vivo* model in which mTERF levels can be manipulated over at least as great a range in a tissue-selective manner. The possibility of redundancy between mTERF and other members of the mTERF family in regulating read-through transcription at the 16S/tRNA-$^{Leu(UUR)}$ gene boundary needs also to be considered.

Modulation of antisense-strand transcripts

We found that alterations in mTERF expression produced systematic changes in the extent of read-through transcription in the antisense direction, as inferred from the relative levels of anti-16S to anti-ND1 transcripts. Increased levels of mTERF, resulting from stable over-expression or from induction of Flp-In™ T-Rex™-293 cells transfected with an mTERF expression construct, shifted the balance of antisense transcripts in the anti-ND1 direction, whereas mTERF knockdown had the opposite effect, shifting the balance in favour of anti-16S. These findings are consistent with the notion that mTERF, bound to its high affinity binding site in the tRNA$^{Leu(UUR)}$ gene, promotes termination of antisense transcription initiated at PL, which has traversed most of circular genome. Increased termination at this site should deplete the representation of anti-16S, whereas decreased termination should increase the amount of anti-16S, consistent with our observations. However, the effects seen are more complex than implied by this simple model. Specifically, the shift towards anti-ND1 under conditions of over-expression consists of a rather drastic decrease in the amount of stable anti-16S, combined with a much smaller decrease in the amount of anti-ND1 (Fig. 3E). Since there are additional, weaker binding sites for mTERF in the IQM tRNA cluster and ND1 coding sequence [16], our finding supports the idea that a high level of mTERF leads to increased occupancy also of these weaker affinity binding sites, restraining readthrough into anti-ND1 as well as the more dramatic effect on readthrough into anti-16S further downstream. On the other hand, mTERF knockdown resulted in a clear decrease in the level of anti-NDI1 but only a small change in anti-16S (Fig. 3F). These findings imply that maintenance of the physiological level of mTERF is important for the formation of stable antisense transcripts of ND1, by preventing readthrough into the rDNA. If this interpretation is correct, one *in vivo* role of mTERF is thus inferred to be the regulation of antisense transcriptional termination, for an unknown physiological reason.

In vitro, mTERF exhibits bidirectional termination activity [15]. If this applies also *in vivo*, it may be that the primacy of post-transcriptional processing, the stabilization of rRNA into ribosomal subunits, and compensatory effects on transcriptional initiation or RNA stability, mask or complicate the effects on sense-strand transcripts. Conversely, antisense transcripts, which are destined only for turnover (or for some unknown physiological function) would appear to be regulated more straightforwardly by mTERF.

A somewhat different interpretation arises from the recent, and thus far unexplained reports of hairpin-loop transcripts deriving from the 16S rRNA gene, whose levels appear to reflect the proliferation status and tumorigenicity of cells [40,41]. It is not yet known how these transcripts arise. Possibilities are that they are created post-transcriptionally by *trans*-splicing or RNA ligation, or else that they arise by template strand-switching during transcription. Our antisense results could thus imply that mTERF influences the rate of their production in ways related to or even independent of its binding to mitochondrial DNA.

Physiological function(s) of mTERF

Given that the effects of mTERF manipulation on the levels of mature mitochondrial transcripts *in vivo* appear to be negated or modified by compensatory mechanisms, it may be that the principal physiological function of this evolutionarily conserved protein is something other than transcriptional regulation as such. In our previous study [16] we speculated that mTERF might play some role in regulating collisions between oppositely moving transcription and replication machineries, facilitating their orderly passage, whilst minimizing the risk

of stalled replication giving rise to recombinogenic 3' ends. A requirement for such activity is well established in both prokaryotic and eukaryotic DNA replication [42,43], and other members of the mTERF family have been inferred to play a role in the completion of DNA replication in human cells [44]. The presence of a transcriptional terminator at a replication pause site moreover provides a potential primer of lagging-strand synthesis commencing immediately from the pausing site, ensuring that no region remains single-stranded and hence susceptible to DNA damage during pausing. The RITOLS model of mtDNA replication [45] postulates that the entire lagging strand is laid down initially as RNA, which might be facilitated by such a mechanism. However, the lagging strand for mtDNA replication is the same strand as the rRNA. Therefore, if bound mTERF were to deliver the 3' end of a paused transcript to an arriving replication complex, this would be as a result of its activity in the sense direction. The role of attenuation on the antisense strand is less clear, although this might provide a primer required for re-initiation of the replication machinery at a stalled replication fork, especially since the former leading strand 3' end may be unavailable, e.g. due to fork regression. A role for DnaG primase in replication restart at stalled, gapped forks has been identified in *E. coli* [46], serving as a precedent for primer-dependent restart. Codirectional collisions between the transcription and replication machineries in *E.* coli also generate leading-strand gaps, with the nascent RNA being recruited as a new primer by the replisome [47].

Another possibility which should be seriously considered is that mTERF's effects on nucleic acid metabolism are incidental to its real biological function inside mitochondria, which may be something completely different. However, arguing against this is the fact that other members of the mTERF family also affect mitochondrial transcript levels, including a recently reported case of the SOLDAT10 protein in *Arabidopsis* chloroplasts, a mutation in which appears to activate retrograde signaling by decreasing plastid rRNA synthesis [48]. MOC1, an mTERF family homologue in *Chlamydomonas*, is required for maintaining mitochondrial RNA levels after exposure to light, although its mechanism of action is unknown and the broader phenotype of the mutant suggests that the effect might be indirect [49].

Conclusions

In summary, our findings support a role for mTERF in influencing mitochondrial transcription *in vivo*, even though it does not appear to set the levels of mature mitochondrial transcripts encoded by the PH1 and PH2 heavy-strand transcription units in a simple manner. It appears to modulate the levels of antisense transcripts, by implication regulating the extent of readthrough by the transcriptional machinery of its high-affinity binding site in the $tRNA^{Leu(UUR)}$ gene, as well as other, weaker mTERF binding sites in the vicinity. Further experiments will now be required to resolve the functional significance of this regulation, and its possible relevance to DNA replication and other processes.

Methods

Cell-lines and cell culture

HEK293T cells and derivatives were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) as previously [16]. HEK293T-derived cell-clones overexpressing natural mTERF were created by recloning the mTERF coding sequence, including its natural stop codon, into the expression vector pcDNA3.1/hygro(-) (Invitrogen) as a *Bam*HI/HindIII fragment. Aliquots of the sequence-verified plasmid DNA (1 μg) were transfected into HEK293T cells using Lipofectamine™ (Invitrogen) diluted in 1 ml of Opti-MEM® (Invitrogen) according to the manufacturer's protocol. Twenty four hours later cells were either harvested (for transient transfection) or placed under hygromycin selection (Calbiochem, 200 μg/ml). Hygromycin-resistant colonies were grown up and tested for expression of the mTERF transgene by reverse transcriptase (RT)-PCR and by electrophoretic mobility shift assay (EMSA) as described in Additional File 1. Flp-In™ T-Rex™ 293 cells transfected with expression constructs for natural mTERF and for epitope-tagged mTERF-MycHis, as well as their induction by doxycycline, were as described previously [16]. mTERF-specific RNA interference was induced by siRNA for 48 h as described previously [16] or by transfection (using Lipofectamine™ 2000, Invitrogen, manufacturer's protocol) with a customized shRNA construct (10 μg) targeting the following sequence within mTERF mRNA (5' to 3'): GCUGUAACUUGAGUACUUU, Open Biosystems Expression Arrest™ pSM2 Retroviral shRNA-mir Library, Oligo ID V2HS_95064 (Thermo Fisher Scientific, Huntsville, AL, USA). shRNA-transfected cells were harvested 48 h after transfection.

Depletion of mitochondrial RNA

Cells were passaged one day before adding ethidium bromide (EtBr) so that the 60 × 15 mm plates were approximately 50% confluent on the day of experiment. EtBr was added to the medium to 250 ng/ml and the cells were incubated for 48 h, after which the plates were approximately 90% confluent. Cells were then passaged at different densities so that each re-seeded plate would reach approximately 70-80% confluence when harvested for RNA extraction. RNA samples were collected before EtBr treatment (day -2), on the day when drug was washed away (day 0) and 24, 48, 72, 96 and

120 h after removing EtBr (days 1-5). To ensure complete removal of EtBr the medium was changed 3 h and 6 h after passaging the cells, and then again every day. Where depletion was carried out in combination with mTERF-directed RNA interference, siRNA transfection was carried out prior to the addition of EtBr (day -2) and was repeated 2 d after removal of the drug (day 2).

RNA extraction, electrophoresis and Northern blotting

Total RNA was extracted from cells using TRIzol® Reagent (Invitrogen) according to the manufacturer's instructions. Any traces of DNA were removed by treatment with RNase-free DNase I (GE Healthcare, manufacturer's recommended conditions), followed by standard acid phenol/chloroform extraction and isopropanol precipitation. For Northern blotting to tRNA probes RNA samples were electrophoresed at 4°C overnight at 100 V in neutral 12% acrylamide/7 M urea gels in TBE buffer, electroblotted onto Zeta-Probe GT membrane (Bio-Rad) at 4°C, u.v.-crosslinked and processed as described previously [50]. Oligonucleotide probes for mitochondrial tRNAs and cytosolic 5S rRNA were radiolabeled using T4 polynucleotide kinase (PNK, MBI Fermentas) according to the manufacturer's protocol and [γ-^{32}P] ATP (Amersham Pharmacia Biotech, 3000 Ci/mmol) and purified using mini Quick Spin Columns (Roche). The probe oligonucleotide sequences were as follows (all 5' to 3'): 5S - GGGTGGTATGGCCGTAGAC, tRNA$^{Leu(UUR)}$ - GTTTTATGCGATTACCGGGC and tRNA^{Phe} - CTAAACATTTTCAGTGTATTGC. Hybridization, washing, autoradiography and phosphorimaging (Phosphorimager SI, Molecular Dynamics) were as described previously [51]. For re-probing, the membranes were stripped by boiling in 0.5% SDS solution for 3 min and cooled to room temperature. For Northern blotting to 16S rRNA or ND1 probes, RNA samples were fractionated on formaldehyde agarose gels and processed for blotting and hybridization as described previously [51], using probes labelled by random-priming [50]. The template used for synthesis of the ND1 probe was as described previously [50]; that for 16S rRNA was the shorter *Apa*I digestion product (230 bp) from the same fragment.

Quantitative RT-PCR

Quantitative RT-PCR was used to estimate the relative amounts of 12S and16S rRNA, ND1 mRNA, cytosolic 18S rRNA and mTERF mRNA. For cDNA synthesis, 5 μg of RNA was reversed transcribed using 40 units of M-MuLV reverse transcriptase (Fermentas), primed by 0.2 μg random hexamers (Pharmacia) in a 20 μl reaction according to manufacturer's instructions. Three dilutions of each cDNA sample (1:10, 1:20 and 1:50) were analysed, and each reaction was performed in three technical replicates. PCR reactions were performed in a LightCycler™ apparatus using LightCycler FastStart DNA Master SYBR Green I kit (Roche) according to the manufacturer's instructions, with the following primer pairs (all 5' to 3') and annealing temperatures: for 18S rRNA, 18Sfor3 - GACGATCAGATACCGTCGTA and 18Srev3 -TGAGGTTTCCCGTGTTGAGT, 52°C; for 16S rRNA, 16Sfor1 - GGTAGAGGCGACAAACCTACCG and 16Srev1 - TTTAGGCCTACTATGGGTGT, 50°C; for ND1 mRNA, ND1for1 - GGCCAACCTCCTACTCC and ND1rev1 - GATGGTAGATGTGGCGGGTT, 50°C. cDNA synthesized from 5 μg of RNA pooled from different cell-lines was used to prepare the standard curve, based on a five-fold dilution series. The homogeneity of all products was checked after each run by melting curve analysis. For strand-specific analysis to distinguish antisense from sense transcripts, 20 pmol of specific primer (TIB MOLBIOL, Berlin, Germany, see Additional File 1, Table S1) were used in the RT step. The PCR step used custom-designed sets of primers and proximity-hybridization probes (TIB MOLBIOL, Berlin, Germany, see Additional File 1, Table S1), with LightCycler (R) FastStart DNA Master HybProbe kit (Roche), according to manufacturer's instructions, and annealing temperatures listed in Additional File 1, Table S1 for each primer pair. The homogeneity of the products was checked after each run by melting curve analysis, according to the annealing temperatures of the hybridization probes as listed in Additional File 1, Table S1. Three dilutions (1:10, 1:20 and 1:50) were analysed from each cDNA. The level of mTERF mRNA relative to 18S rRNA was measured similarly, using hybridization probe sets M1 and C1 (see Additional File 1, Table S1), except that cDNA primed with random hexamers was used as template.

Additional material

Additional file 1: Supplementary text, Table (S1) and Figures (S1, S2, S3). All supplementary data is supplied as a single PDF file containing the following items: Supplementary Methods, Legends to Supplementary Figures, Supplementary Table (Table S1), Supplementary Figures S1, S2 and S3.

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Authors' contributions

AKH performed the experimental work, assisted by MKK for Q-RT-PCR, analyzed the data and co-drafted sections of the manuscript (Results, Materials and Methods, Figure Legends). SKM co-designed the project and co-supervised its initial stages. HTJ co-designed and supervised the project, compiled the figures and drafted the manuscript. All authors saw and approved the final version of the manuscript.

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Effects on mitochondrial transcription of manipulating mTERF protein levels in cultured human HEK293 cells

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ADDITIONAL FILE 1

SUPPLEMENTARY METHODS

Primer and probe sets for Q-RT-PCR using proximity-hybridization probes

See this file, Table S1.

Verification of mTERF transgene expression by RT-PCR

Ten µg of total RNA was used for cDNA synthesis using random hexamers (Pharmacia) and M-MLV reverse transcriptase (Invitrogen) in a 20 µl reaction (manufacturer's recommended conditions). For PCR 2 µl of the RT reaction mix was used in a 25 µl reaction containing 0.4 mM dNTPs (Fermentas), 0.4 µM of both primers and 2 u of Dynazyme DNA polymerase (Finnzymes). Transgene-specific primers were (all 5´ to 3´): (BGH – TAGAAGGCACAGTCGAGGC and mTERF465F – CGAGCAATAACACGTACTCC; 18S specific primers were: 18S-F – TACCTGGTTGATCCTGCCAG and 18S-R – TCGGGAGTGGGTAATTTGC. To exclude possible contaminating DNA, PCR with 18S primers was routinely carried out on each RNA sample, alongside a DNA positive control.

Verification of mTERF transgene expression by EMSA

EMSA was carried out as described previously [16], using probe 'Leu-short' for the mTERF high-affinity binding site, 5 µg of protein in mitochondrial lysate and 5 µg of non-specific competitor poly(dI-dC)–(dI-dC) (Amersham Pharmacia Biotech).

Western blotting to confirm induction of mTERF-MycHis

Western blotting to detect the mTERF-MycHis fusion protein in Flp-In™ T-REx™-293 cells transfected with the mTERF-MycHis construct was performed as previously [16].

Image processing

Western blot and gel images are cropped to show relevant bands. In some cases brightness and contrast were adjusted to make the images optimally visible, but no gamma correction was performed and no other manipulations were done. Non-adjacent tracks of the same gel are shown separately, alongside each other.

SUPPLEMENTARY FIGURE LEGENDS

Figure S1

Manipulation of mTERF expression and effects on steady-state levels of mature mitochondrial RNAs

(A) Verification of expression of mTERF transgene in transfected cell clones, by RT-PCR, using primers specific for the transgene (mTERF 465F and BGH), and for 18S rRNA (18S-F and 18S-R) as loading control, as described in Supplementary Materials and Methods. RNA was extracted from clones, numbered as shown, of hygromycin-resistant cells transfected with the natural mTERF expression construct described previously [16]. – denotes water control, H untransfected HEK293T cells and M the marker ladder. Clone 23 is included here as an example of a clone negative for expression, which was not selected for use in the experiment shown in Fig. 1B. Panels shown alongside each other are aligned, non-adjacent tracks from the same gel. (B) Overexpression of mTERF at the protein level was verified by EMSA using a probe for the high-affinity mTERF binding site, together with mitochondrial protein extracts from clones, numbered as shown, of hygromycin-resistant cells transfected with the mTERF expression construct, plus v – empty vector-transfected cell clone and tr – cells transiently transfected with the mTERF expression construct. – denotes buffer-only control. Clone 7 is included here as an example of a clone negative for expression at the protein level, which was not selected for use in the experiment shown in Fig. 1B. The unbound and bound probe migrated as indicated. The specificity of binding was verified as previously, using cold competitor. Panels shown alongside each other are aligned, non-adjacent tracks from the same (two) gels. (C) Northern blots of RNA samples as indicated, probed for mitochondrial $tRNA^{Phe}$, $tRNA^{Leu(UUR)}$ and cytosolic 5S rRNA as shown. Data from the blots is compiled as Fig. 1E, based on phosphorimaging. The three equivalent samples loaded alongside each are independent RNA preparations from biological replicates.

Figure S2

Effects of mTERF overexpression on mitochondrial transcript levels during EtBr-induced depletion and recovery

Representative series of Northern blots probed successively for mitochondrial $tRNA^{Phe}$ and $tRNA^{Leu(UUR)}$ and for 5S rRNA as indicated. Similar sets of blots were used to compile phosphorimager data for the graphs shown in (B) and (C) and in Fig. 2. RNA was from mTERF overexpresor (OE) clone 3, as characterized in Fig. S1, and the same empty vector-transfected clone as used in the experiment of Fig. S1B. Day -2 indicates sample taken immediately before addition of EtBr to the culture to induce depletion of mitochondrial RNA. Day 0 indicates sample taken 48 h later, immediately after EtBr was washed out from the culture. Days 1-5 indicate the period of subsequent recovery. (B), (C) Relative expression of mitochondrial transcripts in cells overexpressing mTERF, based on phosphorimaging of Northern blots probed successively for mitochondrial $tRNA^{Phe}$, $tRNA^{Leu(UUR)}$ and 5S rRNA. Data (means $\pm$ SD) are ratios of $tRNA^{Phe}$ to 5S rRNA (F/5S) normalized to the ratio at the start of the experiment (time-point -2 d). (B) Cells stably transfected with empty-vector or mTERF overexpression (OE) construct (clone 3, as shown in Fig. S1), sample blots shown in Fig. S2A. (B) Cells treated with mTERF-specific siRNA (or mock-transfected) prior to the addition of EtBr (day -2) and again 2 days after removal of EtBr (day 2). Days 1-5 indicate the period of subsequent recovery. For equivalent data on ratio of $tRNA^{Phe}$ to $tRNA^{Leu(UUR)}$ from the same experiment see Fig. 2. Note that this experiment only measures *changes* in the tRNA ratio during the experiment, but does not allow to extrapolate an absolute ratio of the two tRNAs, since the hybridization efficiency of the two probes may differ. Moreover, to control rigorously for loading differences we used the same blot in each case for reprobing after stripping. Impressionistically, we found nothing to contradict the findings of King and Attardi (*J. Biol. Chem.* 268:10228;1993) that $tRNA^{Phe}$ is expressed normally at a higher level than $tRNA^{Leu(UUR)}$.

Figure S3

Manipulation of mTERF expression affects the relative levels of antisense but not sense transcripts of the 16S rRNA and ND1 genes

(A) Schematic diagram of the 16S rDNA and ND1 region of human mtDNA. For full details of Q-RT-PCR primer sequences and location, see Table S1. The same primer sets were used to assay levels of sense and antisense transcripts, except that reverse transcription step was carried out with the relevant strand-specific primer in each case. (B) Relative steady-state levels of anti-16S and anti-ND1 transcripts, as determined by Q-RT-PCR (primer sets R2 and N2), using hybridization of proximity probes, under various manipulations of the level of mTERF, i.e. the same mTERF overexpressor clone (OE) that exhibited a statistically significant decrease in anti-16S:anti-ND1 ratio using primer sets R1 and N1 (Fig. 3B), compared with cells transfected with empty vector, and HEK293T cells transfected with mTERF-targeted shRNA *versus* mock-transfected cells. In each case, data were normalized to the corresponding control cells. * denotes statistically significant differences from control cells (*t* test, $p < 0.02$). The alterations in the anti-16S:anti-ND1 ratio were qualitatively similar to those obtained in similar experiments using primer sets R1 and N1 (Fig. 3B), although quantitatively slightly lower. (C) As an additional control we validated the earlier findings regarding sense transcripts, using Q-RT-PCR with primer sets R2 and N2 combined with hybridization of proximity probes, under the various manipulations of the level of TERF used in the corresponding experiment on antisense transcripts (Fig. 3B). None of the comparisons showed any significant difference (*t* test, $p > 0.05$). To validate the primer sets and RNA preparations used in the Q-RT-PCR experiments of Fig. 3B and Fig. S3B, C, we conducted a series of controls illustrated by the gels shown in (D). Firstly, the 16S and ND1 primer sets used for reverse transcription and PCR were tested alongside total cell DNA to verify that they gave rise only to a single PCR product of the correct size when cDNA primed with either primer of the set was amplified subsequently amplified with both (panels *i* and *ii*). This was

also checked by melting-curve analysis after PCR and proximity-probe hybridization. Secondly, the purity of each RNA preparation was checked by standard PCR, using the primer pairs employed, plus a primer pair for 18S rDNA, as shown here (panel *iii*), and thus shown to be free of DNA contamination. Thirdly, we excluded that the failure to detect such contaminating DNA was due to inhibition by RNA in the sample, by repeating the analysis after the RNA had been treated with boiled RNase A (panel *iv*). The shRNA construct used in the experiments shown in Fig. 3B and Fig. S3B, C differs from the siRNA used in the earlier experiments. Therefore, prior to use in this context, the effects of transfection of the shRNA were validated using doxycyclin-induced Flp-In™ T-Rex™-293 cells transfected with the tagged mTERF-MycHis expression construct [16] and Western blotting. As shown (E), shRNA constructs mTERF.1 and 5 both effected knockdown, and shRNA mTERF.1 (the construct described under Materials and Methods in the main paper) was selected for use in the experiments shown in Fig. 3B and S3B, C. Note that the knockdown experiments in the main paper, shown in Fig. 3, used normal HEK293T cells, not mTERF-MycHis transfected cells. The latter were used only for initial selection of the appropriate shRNA construct.

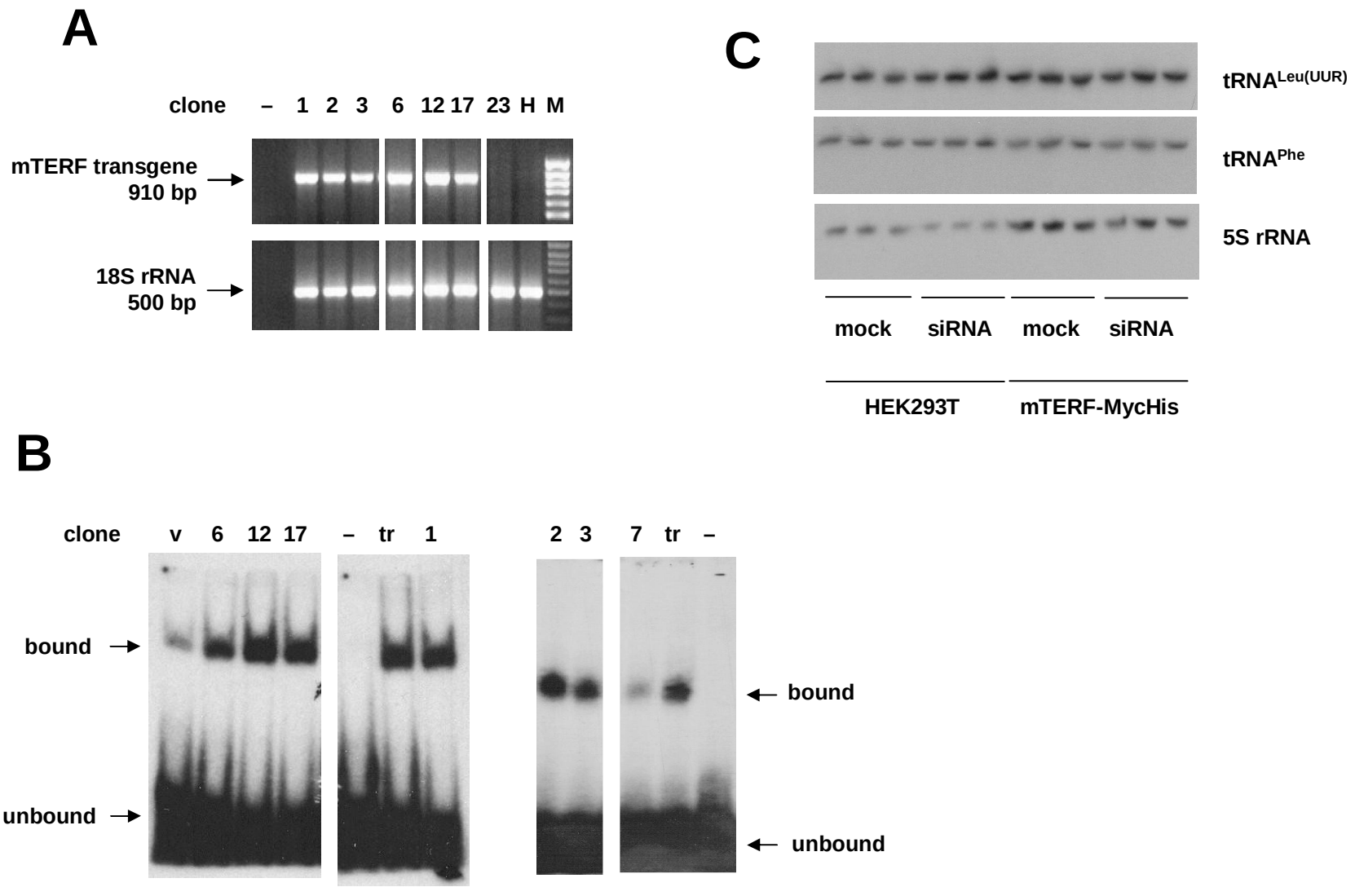
SUPPLEMENTARY TABLES

Table S1

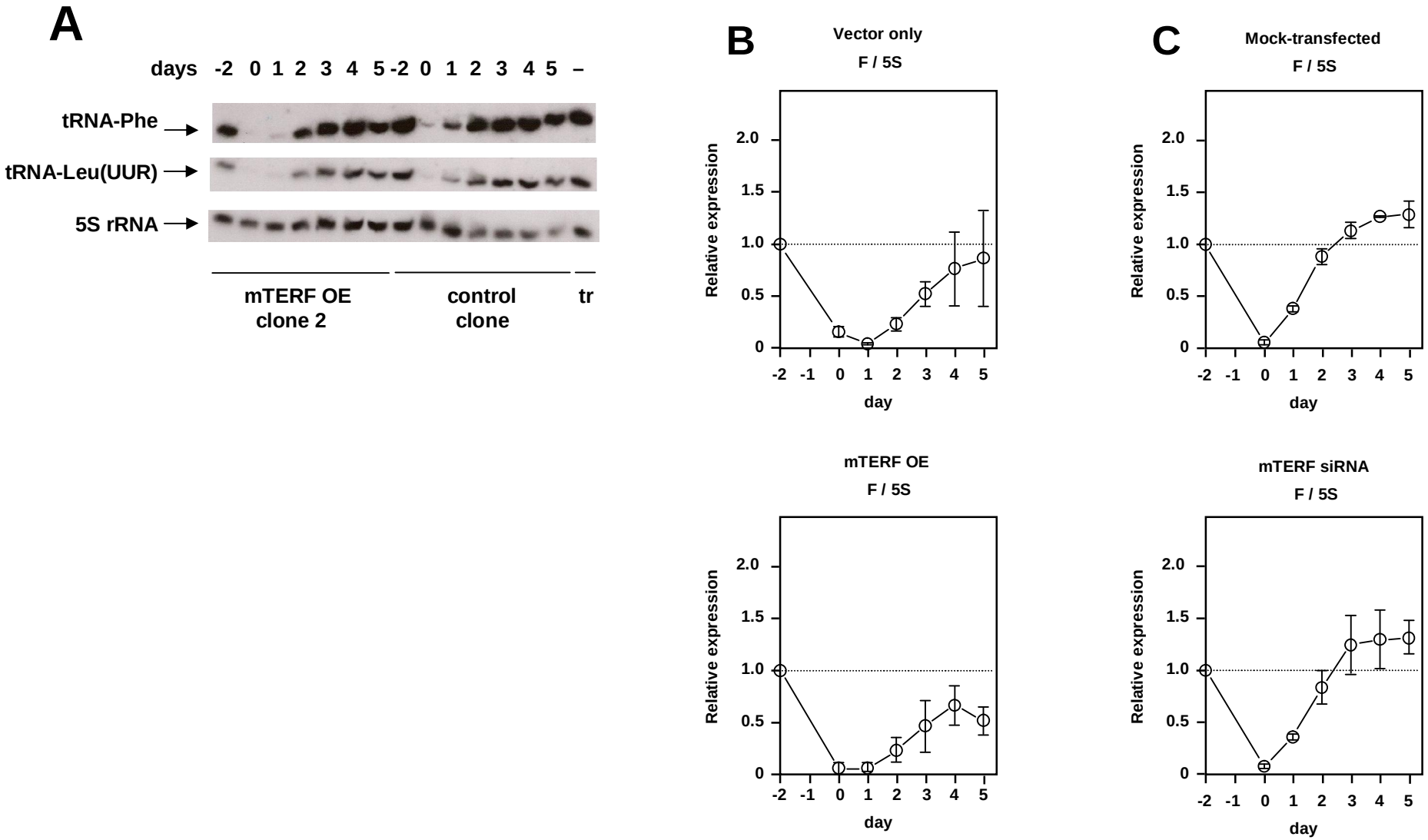
Primer/probe set	**Primer/probe ID, purpose**	**Sequence (5´ to 3´)**	**Annealing Temp (°C)**
R1	Humit16S-fw, primer on antisense template	GGTAGAGGCGACAAACCTACCG	55
	Humit16S-as, primer on sense template	TAGTGGGTGTTGAGCTTGAACG	55
	Humit16S-FL, probe	GGTTCTGTGGGCAAATTTAAAGTTGAACTAAGA-FL	60
	Humit16S-LC, probe	LC640-TCTATCTTGGACAACCAGCTATCACCAGG-P	60
N1	mtND1-se, primer on antisense template	CCAACCTCCTACTCCTCATTGTAC	51
	mtND1-rev, primer on sense template	GATGGTAGATGTGGCGGGTT	51
	mtND1-FL, probe	GGGCCTTTGCGTAGTTGTATATAGCCT-FL	57
	mtND1-LC, probe	LC640-GAATTTTTCGTTCGGTAAGCATTAGGAAT-P	57

R2	16S F, primer on antisense template	AGAGAGTAAAAAATTTAACACCCAT	47
	16S A, primer on sense template	TTCTATAGGGTGATAGATTGGTCC	47
	16S FL, probe	AAGCTCAACACCCACTACCTAAAAAA-FL	55
	16S LC, probe	LC640-CCCAAACATATAACTGAACTCCTCACACC-P	55
N2	ND1_F, primer on antisense template	CCTCATTGTACCCATTCTAATC	45
	ND1_R, primer on sense template	CGTAGTTTGAGTTTGATGCT	45
	ND1_FL, probe	CGCCACATCTACCATCACCCTCTACA-FL	60
	ND1_LC, probe	LC640-CACCGCCCCGACCTTAGCTCT-P	60
T1	12SF101, primer on antisense template	TAGAGGAGCCTGTTCTGTAATCGA	52
	12SB211, primer on sense template	TGCGCTTACTTTGTAGCCTTCAT	52
	12S FL, probe	CGATCAACCTCACCACCTCTTGCTC-FL	60
	12S LC640, probe	LC640-CCTATATACCGCCATCTTCAGCAAACCC-P	60

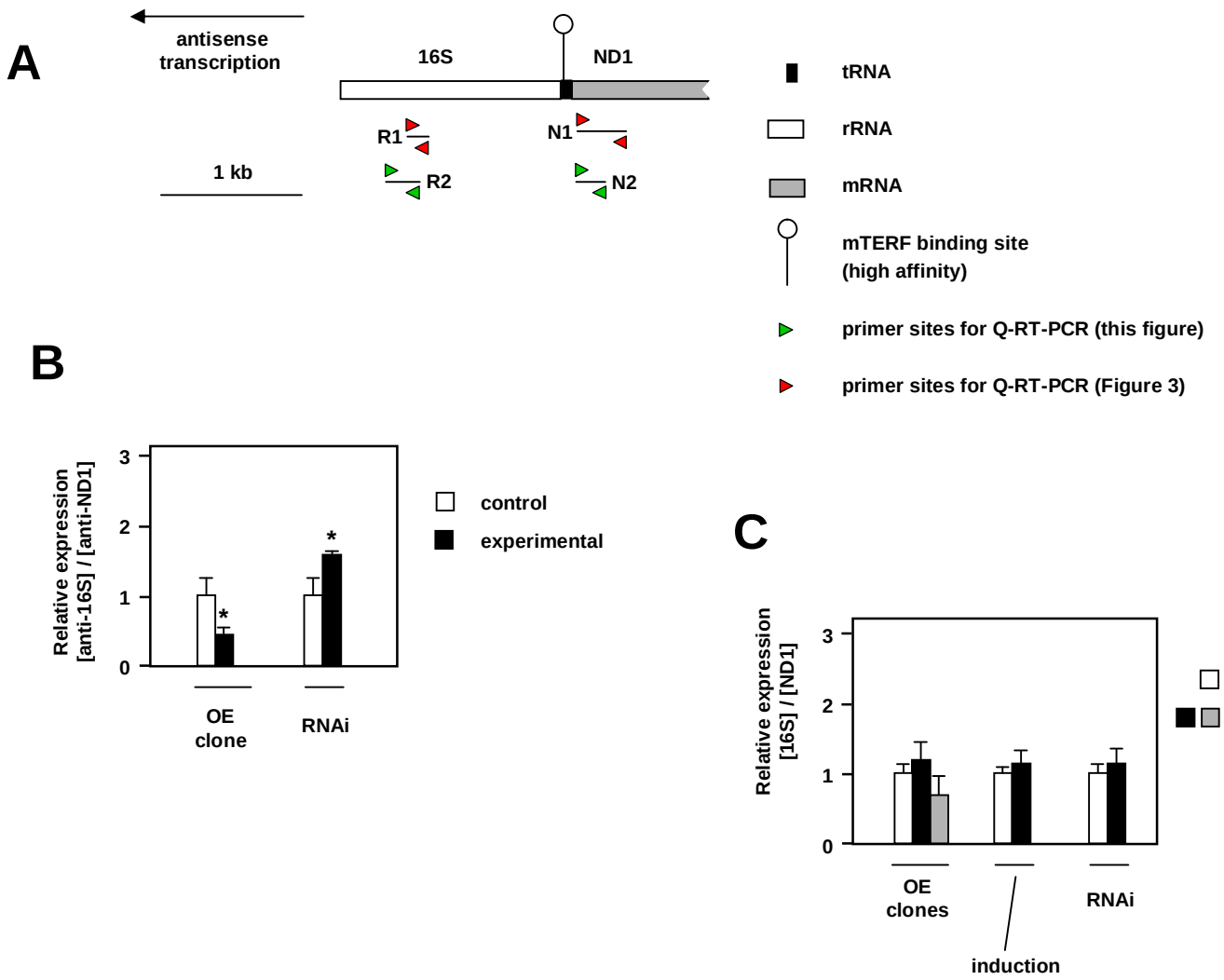
M1	MTERF S, primer for sense strand	GCAGAGCCTTTCCTTAGGAC	50
	MTERF A, primer for antisense strand	GTCATCCAACATCTTGAACCAA	50
	MTERF FL, probe	AGGTTTCCTGGTGCCATAATGGT-FL	56
	MTERF LC, probe	LC640-AGGTAGTTCAAACCTTTTGAAATGCTTGT-P	56
C1	18S for, primer on antisense template	ACGRACCAGAGCGAAAGCAT	52
	18S rev, primer on sense template	GGACATCTAAGGGCATCACAGAC	52
	18S FL, probe	TCGGAACTACGACGGTATCTGATCGTC-FL	59
	18S LC, probe	LC640-CGAACCTCCGACTTTCGTTCTTGAT-P	59



Hyvärinen et al., Figure S1



Hyvärinen et al., Figure S2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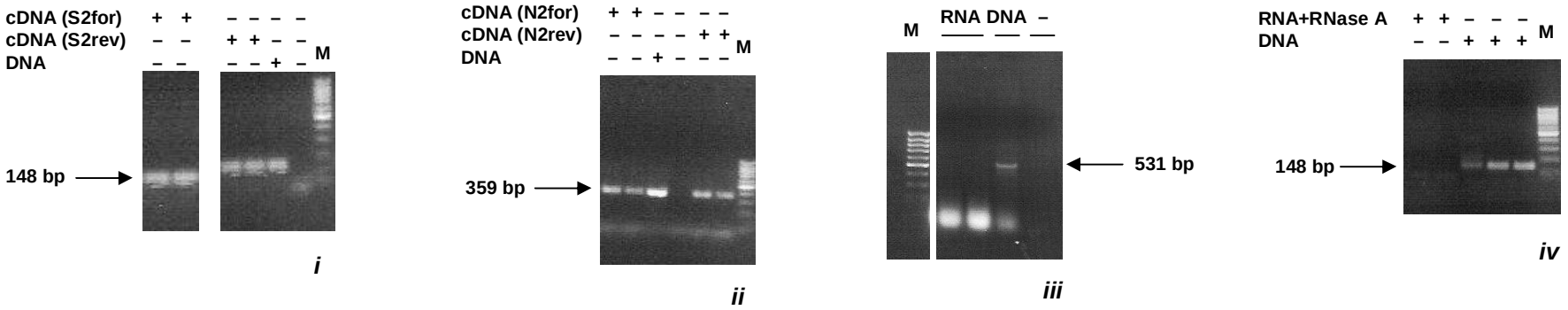
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cDNA (N2rev) – – – – + +
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RNA DNA –
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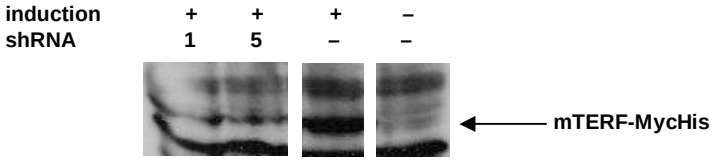
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doi:10.1093/nar/gkl703

Alterations to the expression level of mitochondrial transcription factor A, TFAM, modify the mode of mitochondrial DNA replication in cultured human cells

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ABSTRACT

Mitochondrial transcription factor A (TFAM) is an abundant mitochondrial protein of the HMG superfamily, with various putative roles in mitochondrial DNA (mtDNA) metabolism. In this study we have investigated the effects on mtDNA replication of manipulating TFAM expression in cultured human cells. Mammalian mtDNA replication intermediates (RIs) fall into two classes, whose mechanistic relationship is not properly understood. One class is characterized by extensive RNA incorporation on the lagging strand, whereas the other has the structure of products of conventional, strand-coupled replication. TFAM overexpression increased the overall abundance of RIs and shifted them substantially towards those of the conventional, strand-coupled type. The shift was most pronounced in the rDNA region and at various replication pause sites and was accompanied by a drop in the relative amount of replication-termination intermediates, a substantial reduction in mitochondrial transcripts, mtDNA decatenation and progressive copy number depletion. TFAM overexpression could be partially phenocopied by treatment of cells with dideoxycytidine, suggesting that its effects are partially attributable to a decreased rate of fork progression. TFAM knockdown also resulted in mtDNA depletion, but RIs remained mainly of the ribosubstituted type, although termination intermediates were enhanced. We propose that TFAM influences the mode of mtDNA replication via its combined effects on different aspects of mtDNA metabolism.

INTRODUCTION

In mammalian cells, mitochondrial DNA (mtDNA) was long believed to replicate by an unusual, strand-asymmetric mechanism (1). However, recent studies, using two-dimensional neutral agarose gel electrophoresis (2DNAGE), have revealed the presence, both in vertebrate tissues and cultured cells, of two classes of mtDNA replication intermediates (RIs) whose structures are not consistent with the strand-asymmetric model. Both classes are essentially duplex throughout their length, but differ in their ribonucleotide content (2,3). One class shows **e**xtensive **R**NA **i**ncorporation **o**n the **l**agging **s**trand [ERIOLS, Ref. (2)], whereas the other has structures fully consistent with conventional, strand-coupled DNA replication (3–5). ERIOLS intermediates are generally nicked or gapped on the RNA strand (2) and are hence labile to partial degradation during extraction.

The mechanistic relationship between RIs of the ERIOLS and strand-coupled types, as well as how they relate to the 'orthodox', strand-asymmetric replication model, are not properly understood. ERIOLS intermediates have been suggested to be processed to resemble those of the strand-coupled type via a maturation step (2). Different replication modes may also operate simultaneously in the same cell. In solid tissues, strand-coupled replication appears to initiate bidirectionally in a broad origin zone, spanning at least several kilobases downstream of the major non-coding region (NCR) of the genome (5,6). In cultured cells recovering from drug-induced mtDNA depletion, such initiation is confined to a much narrower region of the NCR (3). The initiation mechanism which gives rise to RIs of the ERIOLS type remains unclear. Initiation within the NCR can also give rise to the synthesis of 7S DNA (1), which establishes the characteristic D-loop form of mtDNA, although its relationship with productive replication of the genome remains enigmatic.

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The mitochondrial transcription factor TFAM, an abundant HMG-box protein of mitochondria, appears to have multiple functions in mtDNA metabolism (7). It was originally characterized by the absolute requirement for the protein for transcriptional initiation *in vitro* at either the heavy- or light-strand promoter of the genome (8). More recently, these findings were confirmed using a fully reconstituted system, containing mtDNA-derived templates, RNA polymerase and the additional transcription factor TFB1M or TFB2M (9). Transcription from the light-strand promoter is required to create the primer for heavy-strand mtDNA synthesis according to the orthodox, strand-asymmetric replication model. Therefore TFAM has been considered to be an essential protein also for mtDNA replication. Consistent with this view, abolition of TFAM expression using a conditional knockout strategy in the mouse showed clearly that TFAM is required for mtDNA maintenance as well as cellular function and survival (10). However, this finding is also consistent with TFAM protein having other essential roles in mtDNA metabolism.

TFAM has been proposed to play a structural role in the maintenance of the mitochondrial chromosome, independent of its transcriptional activity. It is highly abundant, is mainly (or entirely) complexed with mtDNA in nucleoid structures (11,12), shows significant, non sequence-specific DNA-binding (8) and promotes DNA compaction (13), leading to the suggestion that it coats the entire DNA in a manner similar to histones in the eukaryotic nucleus or the HU protein in bacteria (14). Its homologue in yeast, Abf2p, has been shown to induce compaction by introducing sharp bends into the DNA backbone (15) and is required for the stable maintenance of wild-type mtDNA (16).

Mammalian TFAM has preference for binding to branched DNA structures such as Holliday junctions (17) and to cisplatin-damaged or oxidized DNA (18). *In vitro*, TFAM promotes the resolution of D-loop forms (19). It also interacts physically with p53 [Ref. (20)], suggesting a possible function in DNA repair or other recombinational processes. Although it does not have a directly protective role, TFAM overexpression in rat myoblasts has been reported to accelerate the recovery of mtDNA levels after peroxide damage (21) and transgenic expression of human TFAM in mice mitigates mtDNA loss and other mitochondrial defects after cardiac ischemia (22). In yeast, Abf2p is required for recombination and segregation of mtDNA to daughter cells (23) and genetic evidence also implicates it in non-recombinational mtDNA repair pathways (24). All of these pieces of evidence point to TFAM and its homologues being key regulators of DNA transactions in mitochondria.

In organello, TFAM imported into rat liver mitochondria stimulates the synthesis both of mitochondrial RNAs (25) and 7S DNA (26). Transient overexpression of TFAM in cultured HEK cells also results in increased transcription, but with no change in mtDNA copy number (27). However, high levels of TFAM added exogenously *in vitro* (9,28,29), as well as prolonged over expression in HEKcultured cells (27), bring about a paradoxical decrease in transcription. This effect may be attributable to an over-condensed state in the template DNA. The transient increase in mitochondrial transcription brought about by TFAM over-expression in HEK cells is accompanied by an increased level of RNase H-sensitive mtDNA species (27), which may correspond with RIs of the ERIOLS type.

Several lines of evidence suggest that TFAM regulates mtDNA copy number independently of its role(s) in transcription. Heterozygosity for TFAM knockout produces copy number depletion of ~40% in mice (10) and 50% in chicken cells (30), but with minimal effects on RNA levels. Although human TFAM has only a weak transcription-stimulatory effect on mouse mtDNA promoters *in vitro*, transgenic expression of human TFAM in mice produces a stoichiometric increase in mtDNA levels (31). Overexpression of a transcriptionally inert variant of TFAM in human cells also results in a proportionate copy number increase (14), whilst TFAM knockdown by RNA interference (RNAi) causes copy number depletion with only minor effects on transcription per template molecule (14). In chicken cells in which the endogenous c-TFAM gene has been disrupted, transgenic c-TFAM lacking the C-terminal tail region required for transcription-promoting activity is nevertheless able to support maintenance of mtDNA at 50% of wild-type levels (30).

The idea that TFAM regulates mtDNA copy number by a simple titration model is, however, contradicted by the observation that, following transient, ethidium bromide (EtBr)-induced mtDNA depletion in cultured cells, TFAM levels were observed to recover more slowly than mtDNA. This suggests that the packing ratio of TFAM on mtDNA can vary and may influence the rate of mtDNA replication (32).

In order to investigate further the effects and mode of action of TFAM on mtDNA replication and copy number modulation, we analysed mitochondrial RIs from cells overexpressing TFAM and from cells in which TFAM expression was knocked down by RNAi. We report here that overexpression of TFAM brings about a dramatic change in the relative abundance of strand-coupled versus ribosubstituted (ERIOLS-type) RIs, accompanied by systematic alterations in copy number, transcript levels and mtDNA topology. In contrast, TFAM knockdown results in copy number depletion, but with only minimal effects on mtDNA RIs. The findings are consistent with the idea that the synthesis of mtDNA is dependent on at least two different TFAM-influenced processes, one of which is transcription-associated, the other related to mtDNA organization.

MATERIALS AND METHODS

TFAM constructs

Full-length *TFAM* cDNA lacking its usual stop codon was amplified from a cDNA clone (27, kind gift of Dr R. Wiesner), using the following primers (restriction sites as indicated, underlined, start codon in bold italics):

5′-CCGGAATCCGCG***ATG***GCGTTTCTCCGAAGC-3′ (EcoRI) and 5′-CGCGGATCCACACTCCTCAGCACCATATTTTCG-3′ (BamHI)

The restriction-digested PCR product was ligated into EcoRI + BamHI-cut pcDNA3.1(−)Myc-HisA (Invitrogen) to create the construct mtTFA-myc, capable of directing the expression of C-terminally Myc-His epitope-tagged TFAM. The full-length TFAM cDNA, including the natural stop codon, was amplified using the following primers

(restriction sites as indicated, underlined, plus start and stop codons in bold italics):

5′-CCCAAGCTTGCG***ATG***GCGTTTCTCCGAAGC-3′ (HindIII) and 5′-CGCGGATCC***TTA***ACACTCCTCAGCAC-CATATTTTC-3′ (BamHI).

The restriction-digested PCR product was ligated into HindIII + BamHI-cut pcDNA3.1(+) vector, to create the construct mtTFApcDNA3.1 for transient expression.

In order to create cell lines inducibly expressing either natural TFAM or C-terminally Myc epitope-tagged TFAM, the two plasmids described above were digested with PmeI and the liberated inserts recloned into the vector pcDNA5/FRT/TO (Invitrogen), which was then transfected into the Flp-In™ T-Rex™-293 host cell line (Invitrogen) according the manufacturer's recommendations. Full details of the use of this system to create cell lines inducibly expressing proteins involved in mtDNA metabolism will be published elsewhere (S. Wanrooij *et al*., manuscript in preparation).

siRNAs for TFAM knockdown

TFAM siRNAs were synthesized using the *Silencer*™ siRNA construction kit (Ambion). Six putative TFAM-specific siRNA sequences were selected using the manufacturer's prediction programme (www.ambion.com/techlib/misc/siRNA_finder.htlm). After testing by transfection and western blotting (see below), two were found to be efficient. The sequences of the relevant mRNA targets were as follows. Si2: 5′-AAGTTGTCCAAAGAAACCTGT-3′ (np 273–293 of the TFAM mRNA sequence, Genbank NM_003201) and Si4: 5′-AAGATGCTTATAGGGCGGAGT-3′ (np 431–451, exon 4). See legend to Supplementary Figure 3 for details of other siRNAs tested in trial experiments.

Cell-culture and transfection

HEK293T and Flp-In™ T-Rex™-293 cells (Invitrogen) were cultured in DMEM containing 4.5 g/l glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μg/ml uridine and 10% fetal bovine serum, at 37°C in a humidified atmosphere with 5% CO_2 in air. No antibiotics were added for HEK293T cells, but transgenes in Flp-In™ T-Rex™-293 cells were maintained under selection with hygromycin and blasticidin according to the manufacturer's recommendations. All cell lines were routinely detached by pipetting alone and passaged at 1:10 dilution every 3–4 days. TFAM transgene expression was induced by adding 10 ng/ml doxycyclin (Sigma-Aldrich) to the culture medium for the times indicated in the Figures and legends. Transfections were carried out using TransFectin™-lipid reagent (Bio-Rad), following the manufacturer's recommended procedure, with 12 μg of plasmid DNA and 40 μl of reagent, both in 1.5 ml of serum-free medium, per 10 ml plate.

For TFAM knockdown, HEK293T cells were transfected using Lipofectamine™ 2000 (Invitrogen) and a final concentration of 20 nM of siRNA. To arrest mtDNA synthesis by chain termination, cells were treated for various times in medium containing 100 μM dideoxycytidine (Sigma-Aldrich). To suppress both mtDNA copy number and mitochondrial transcription, cells were treated with medium containing EtBr (50 ng/ml) for 72, after which cells were washed and replated in fresh medium, then cultured for a further 48 h.

DNA and RNA extraction and quantitation

For mtDNA copy number analysis, total cellular DNA was extracted using standard techniques (33). Copy number was assessed independently by two different methods, to minimize possible artefacts. For Southern blotting, total DNA was cut by EcoRI and analyzed as described in Ref. (34), with quantitation by phosphorimaging (Storm 840 scanner and ImageQuant 5.1 software, all from Molecular Dynamics). Copy number was also estimated by real-time quantitative PCR (35) with Taqman probes for mitochondrial cytochrome *b* and for amyloid precursor protein (APP), used as a single-copy nuclear DNA standard. Primers and probes were as follows (all 5′–3′): APP Forward: TTTTTGTGTGCTCTCC-CAGGTCT, APP Reverse: TGGTCACTGGTTGGTTGGC, APP Probe (FAM+BHQ): CCCTGAACTGCAGATCACCA-ATGTGGTAG, Cyt-b Forward: GCCTGCCTGATCCTCC-AAAT, Cyt-b Reverse: AAGGTAGCGGATGATTCAGCC, Cyt-b Probe (TET+BHQ): CACCAGACGCCTCAACC-GCCTT.

RNA extraction from cells, agarose or urea–PAGE and Northern hybridization were as described previously (34,36). Probes were ^{32}P end-labelled oligonucleotide as follows (5′–3′): for ND3 mRNA, GTCACTCATAGGCCAG-ACTT, for 5S rRNA (loading control), GGGTGGTATGGC-CGTAGAC, for $tRNA^{Leu(UUR)}$ and $tRNA^{Tyr}$ as described previously (37). Quantitation was by phosphorimaging as for mtDNA copy number.

For the preparation of mtDNA (mitochondrial nucleic acids) for analysis of RIs, mitochondria were isolated from cells essentially as described by Spelbrink *et al*. (38). Briefly, cells from 10 to 20, 14 cm plates were collected by pipetting in PBS, centrifugation at 400 g_{max} for 3 min at room temperature and transfer to ice. The cell pellet was resuspended by gentle pipetting in two volumes of ice-cold 0.1× homogenization buffer (4 mM Tris–HCl, pH 7.8, 2.5 mM NaCl, 0.5 mM $MgCl_2$), kept on ice for 5 min and homogenized in a glass homogenizer with 20 strokes of a tight-fitting pestle. Disruption of the cells was monitored by microscopy. One-ninth volume of 10× homogenization buffer was added and nuclei and cell debris were pelleted by sequential centrifugations at 1200 g_{max} for 3 min at 4°C until no pellet was visible. Mitochondria from the post-nuclear supernatants were recovered by centrifugation at 16 000 g_{max} for 10 min at 4°C. The mitochondrial pellet was washed once in resuspension buffer (10 mM Tris–HCl pH 7.4, 0.32 M sucrose, 1 mM EDTA, 5 mM $MgCl_2$) and re-centrifuged at 16 000 g_{max} for 10 min at 4°C. The pellet was placed immediately on ice and resuspended thoroughly in 500 μl of DNA extraction buffer (25 mM EDTA pH 8.0, 75 mM NaCl) followed by the addition of 50 μl 10% SDS with gentle mixing and incubation on ice for a further 10 min. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, pH 8,0) was added and the tube was shaken gently overnight at 4°C on a rotatory shaker, then centrifuged at 5000 g_{max} for 15 min at 4°C. The aqueous phase was transferred to a fresh tube and phenol extraction repeated several times until the interface was clear, after which 0.2 vol. 10 M ammonium acetate and 2 vol. 80% EtOH were added to the final aqueous phase. The solution was gently mixed, incubated on ice for 15 min and centrifuged at 5000 g_{max} for 15 min at 4°C. The precipitated nucleic acids were washed once with 80%

EtOH and centrifuged at 5000 g_{max} for 5 min at 4°C min. The pellet was air- dried, dissolved in 80 µl of TE buffer and stored at 4°C.

Enzymatic treatment of DNA

MtDNA samples were treated with the following DNA-modifying enzymes under conditions recommended by the manufacturers: T7 gp3 endonuclease (New England Biolabs), topoisomerase I (New England Biolabs) and topoisomerase IV (John Innes Enterprises).

Two-dimensional neutral agarose gel electrophoresis

One microgram of total mitochondrial nucleic acids was used per analysis. Restriction digestions were performed following manufacturers' recommendations, except for BclI which was carried out at 37°C for double the usual reaction time. If subsequent nuclease treatments were used, DNA was first recovered by ethanol precipitation and resuspended in the appropriate reaction buffer, before treatment with 50 U of RNaseI (New England Biolabs), 2 U of RNaseH (Promega), each for 1 h at 37°C or 50 U S1 Nuclease (Promega) for 30 s. Reactions were stopped by the addition of an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1, pH 8.0) and immediately extracted. 2DNAGE was performed essentially as described in Kajander *et al*. (39). The first-dimension was run without EtBr in a 0.4% agarose gel in TBE buffer, 1.2 V/cm for 24 h at 4°C. The gel was stained with EtBr (300 ng/ml) in TBE. Individual lanes were cut out, rotated 90° and 1.0% agarose containing 300 ng/ml EtBr, precooled to 55°C, was cast around them. The second dimension was run at 6 V/cm for 5 h at 4°C with constant buffer recirculation. For analysis of high molecular weight fragments (e.g. 16.6 kb mtDNA linears), the first-dimension gels were 0.28% agarose, run at 1.4 V/cm for 24 h at room temperature, with the second dimension in 0.58% agarose, 300 ng/ml EtBr, run at 2.6 V/cm for 67 h at room temperature with constant buffer recirculation. Gels were processed for Southern blotting using standard procedures.

Radiolabelled probes and blot hybridization

For Southern hybridization, the following probes were created by *Pfu*-PCR, using cloned segments of human mtDNA as template and subsequently sequenced to confirm their identity: O_H (np 35–611, Anderson *et al*., 1981), ND2 (np 4480–4988), A8-6 (np 8460–9107), ND4 (np 11 161–11 640) and ND5 (np 12 992–13 670). Probes were labelled using Rediprime™ II random prime labelling kit (Amersham) and [α-^{32}P]dCTP (Amersham; 3000 Ci/mmol).

Sub-fractionation of mitochondria

For assaying the localization of recombinant TFAM the mitochondrial pellet, prepared as above, was resuspended in 2 vol. of lysis buffer (0.25 M sucrose, 20 mM Tris–HCl, pH 7.6, 2 mM EDTA, 7 mM β-mercaptoethanol). Mitochondria were lysed by adding 20% NP40 to a final concentration of 0.5% (v/v) with incubation on ice for 1 h. After centrifugation at 16 000 g_{max} for 10 min at 4°C the pellet and supernatant fractions were processed for SDS–PAGE.

SDS–PAGE and western blotting

SDS–PAGE used 7.5–12% polyacrylamide (Laemmli) gels under standard conditions. Sample preparation, western blotting and immunodetection were carried out as described previously (38). Primary antibodies used were: mouse anti-Myc monoclonal 9E10 (Roche Molecular Biochemicals), 1:15 000 dilution of a 5 mg/ml stock, rabbit anti-human TFAM (kind gift of Dr R. J. Wiesner), 1:10 000 dilution and mouse anti-α-actinin monoclonal AT6/172 (Upstate) 1:5000 of a 1 mg/ml stock. Signals were quantified using a ChemiDoc XRS chemiluminescence detection instrument and associated QuantityOne software.

RESULTS

Effects on mitochondrial nucleic acids of modulating TFAM expression levels *in vivo*

We manipulated TFAM expression levels in cultured human cells using transient or inducible expression of epitope-tagged or untagged TFAM, as well as TFAM knockdown by RNAi (Figure 1, see also Supplementary Figures 1–3). Transient expression (data not shown) produced essentially the same effects on all parameters studied as inducible over-expression using the Flp-In™ T-Rex system, although with less quantitative reproducibility. Inducible over-expression of TFAM carrying a C-terminal MycHis tag produced qualitatively similar but quantitatively more dramatic effects, namely a small but transient increase in mtDNA copy number, followed by progressive mtDNA depletion. After 10 days of full induction (10 ng/ml doxycyclin, full details on the use of the induction system to be published elsewhere, Wanrooij *et al*., manuscript in preparation), TFAM-MycHis expression resulted in the reduction of mtDNA levels to ~20% of control levels. Over-expression of TFAM with its normal stop codon (TFAM-stop) caused a decrease in mtDNA copy number of 40–60%. Copy number depletion was verified by two independent methods, real-time PCR and phosphorimaging of Southern blots (Supplementary Figures 1 and 2).

Although the absolute amount of TFAM protein did not increase markedly during induction, measured relative to a loading control (Supplementary Figures 1 and 2), the progressive drop in mtDNA copy number means that the amount of TFAM protein per mtDNA molecule did increase substantially and consistently during induction: in the case of TFAM-stop to over twice the endogenous level, in the case of TFAM-MycHis by an order of magnitude. Crude fractionation of mitochondrial protein lysates by sucrose density-gradient centrifugation (Supplementary Figure 2) showed that over-expressed TFAM-MycHis partitioned, like endogenous TFAM, mainly into the pellet fraction, forming high-molecular weight complexes in the same proportion as endogenous TFAM, indicating that it is likely complexed with mtDNA.

Previous experiments *in vitro* and *in organello* have suggested that a large excess of TFAM can suppress rather than activate transcription. We measured the steady-state levels of various mitochondrial transcripts using northern blot analysis (Supplementary Figures 1 and 2 and other

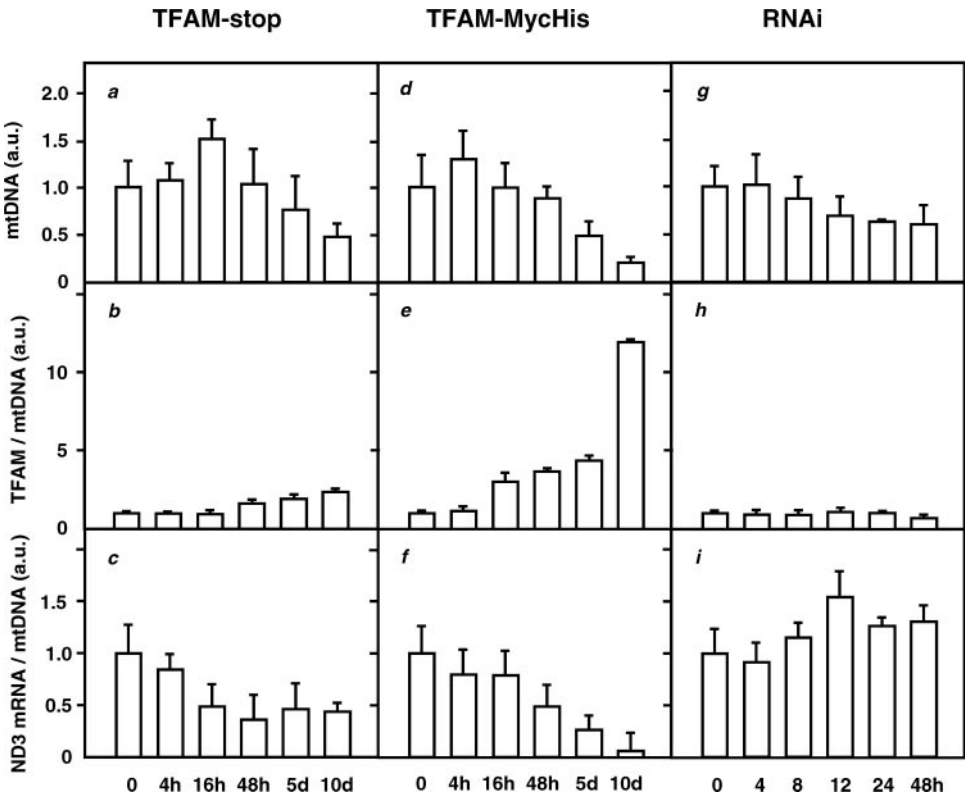

Figure 1. Effects of induced expression of TFAM-stop and TFAM-MycHis and of RNAi knockdown of TFAM expression. Mitochondrial proteins, DNA and RNA were analysed from Flp-In™ T-Rex™ -293 cells stably transfected with the TFAM-stop (a–c) or TFAM-MycHis construct (d–f), induced over the times indicated or from HEK293T cells (g–i) following transfection with siRNAs Si2 and Si4 over the times indicated. In each case, error bars indicate means ± SEs from at least three independent experiments. a.u., arbitrary units. Measurements of mtDNA levels (a, d and g) are arbitrarily normalized to the mean values for uninduced or untreated cells. For cells under TFAM induction, the measurements were made by two independent methods, Southern blotting and Q-PCR and the values plotted for each time point are the means of measurements by the two methods, shown in Supplementary Figures 1 and 2. (**b**, **e** and **h**) show TFAM protein levels normalized to the mtDNA levels shown in (**a**, **d** and **g**), then normalized against the level in uninduced or untreated cells. (**c**, **f** and **i**) show ND3 mRNA levels normalized first against the 5S rRNA loading control, then against the mtDNA levels shown in (a, d and g), then finally against the level in uninduced or untreated cells. Samples of the raw data and compiled data for TFAM protein, mtDNA and RNA levels on which this figure is based, are shown in Supplementary Figures 1–3.

data not shown), under conditions where over-expression of TFAM-stop or TFAM-MycHis was induced *in vivo*. The level of short-lived mRNAs such as ND3 showed a marked decline even relative to the decreased amount of mtDNA. Although modulation of post-transcriptional processing and RNA stability contribute to changes in the steady-state level even of short-lived transcripts, the drop in ND3 mRNA levels is consistent with a substantial drop in transcription per template molecule, under conditions of TFAM over-expression, which was especially marked for TFAM carrying the C-terminal MycHis tag. As a more rigorous and direct test of its effects on transcriptional activity, we also analysed the consequences of TFAM-MycHis expression on the rate of recovery in mitochondrial tRNA levels in cells treated for 72 h with EtBr, following removal of the drug (Supplementary Figure 4).

In conformity with the published literature, RNAi knockdown of TFAM produced a progressive reduction of TFAM protein levels and mtDNA copy number. A combination of two siRNAs was selected, based on preliminary trials, which decreased TFAM protein to low levels (<10%) during 7 or 14 days continuous culture (Supplementary Figure 3). Over shorter-time periods, both TFAM protein and mtDNA depletion by RNAi were rather modest and the levels of TFAM protein or ND3 mRNA per template mtDNA molecule were almost unchanged from control cells (Figure 1).

Altered TFAM expression leads to systematic effects on mtDNA replication intermediates

The copy number depletion of mtDNA produced either by RNAi knockdown or by over-expression prompted us to investigate further the effects of these treatments on mtDNA replication, using 2DNAGE. Transient expression of TFAM-stop or inducible overexpression of either TFAM-stop or TFAM-MycHis, produced dramatic and systematic effects on the patterns of mtDNA RIs, which were essentially the same in all three cases (Supplementary Figure 5b). The most consistent effects on RIs were seen after 48 h of induced over-expression (or 48 h after transient transfection), when effects on mitochondrial transcripts were clearly evident and quite similar in all cases.

TFAM overexpression resulted in a substantially increased abundance of RIs relative to the unit-length restriction fragment (Figure 2; see Supplementary Figure 5 for further explanations and interpretations of the gel data). For example,

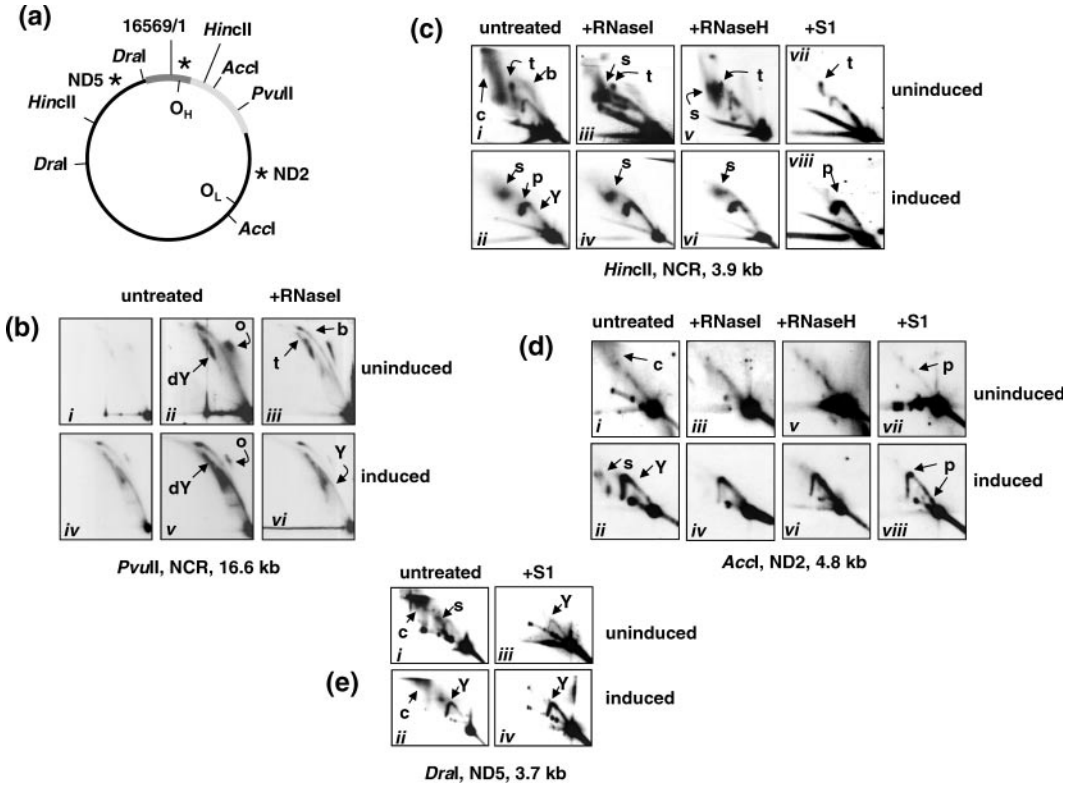

Figure 2. 2DNAGE analysis of mtDNA replication intermediates (RIs) in cells induced to overexpress TFAM-stop. (**a**) Diagrammatic map of human mtDNA, showing the origins of heavy- (O_H) and light-strand (O_L) replication according to the orthodox model, relevant restriction sites and probes for the three regions of the genome analysed (approximate location of probes indicated by asterisks). NCR shown as dark grey bar, rDNA as pale grey bar. (**b**–**e**) 2DNAGE of mtDNA from uninduced cells and from cells induced to express TFAM-stop for 48 h, analysed using the restriction digests and probes indicated, with or without additional enzymatic treatments as shown. In each panel, the various arcs and other salient features are denoted as follows: Y, standard Y arcs, dY, standard doubleY arcs, c, 'cloud' of RNase-sensitive material, o, circular molecules, b, standard bubble arcs, s, slow-moving Y-like arcs, sensitive to various nucleases, t, termination intermediates lying on a portion of a standard X arc, p, prominent pause sites. See Supplementary Figure 5 for diagrammatic interpretations of the various arcs. Panels i and iv of part (b) and panels iii and iv of part (c) are equivalent exposures, for comparison. Other panels of uninduced cell mtDNA are 5- to 10-fold more exposed than induced cell material, in order to reveal the main features of the arcs. Note the general enhancement of RIs, relative loss of nuclease-sensitive species, of termination intermediates and of bubble arcs, following TFAM induction. The appearance of a complete or almost complete, Y arc in Figure 2b, panels iv–vi, is consistent with frequent strand breakage at O_H or with recombinational strand-switching (also generating a free end at O_H) or with frequent initiation far distant from the NCR.

using a restriction enzyme which cuts only once in the genome (PvuII, np 2560, Figure 2b), TFAM over-expression gave a clear enhancement of the clubheaded bubble arc indicative of initiation far upstream of the restriction site, as well as revealing a prominent simple Y-arc. In uninduced cells these RIs were either much fainter or absent. The main features were the clubheaded bubble arc and corresponding double-Y arc, as well as a number of species that were sensitive to or modified by, RNaseI treatment. The RNaseI-sensitive species included circular molecules, a short arc which corresponds with dimeric circles and/or broken theta forms (T. Yasukawa, personal communication), plus a diffuse cloud of material migrating in the region between circular molecules and the bubble arc. A putative termination arc was also visible only in uninduced cells, although this was revealed more clearly when other species were modified by RNaseI (Supplementary Figure 5).

In other digests, the exact transformations of RIs resulting from TFAM overexpression varied according to which region of the genome was being analysed (Figure 2c–e). In general, ERIOLS-type RIs, i.e. slow-moving arcs and 'clouds' of heterogeneous, nuclease-sensitive material were diminished and migrated as more discrete entities. There was general enhancement of nuclease-resistant arcs, in particular those resistant to S1 nuclease. In addition, rather specific subtypes of RIs were seen to accumulate along the arcs, which differed from those seen in uninduced or untransfected cells.

Changes were most dramatic in the rDNA region, extending to O_L (Figure 2d). In uninduced cells, in contrast to the patterns of RIs from this region of the genome seen in solid tissues or in cultured cells recovering from drug-induced mtDNA depletion (2–4), complete Y-arcs of the strand-coupled type were not detectable, even on long exposure. Instead, a heterogeneous 'cloud' of complex, high-molecular weight material was seen, plus the ascending portion of a Y-like arc, ending in a faintly detected replication pause site within the ND1 gene. These forms were partially sensitive to or modified by nucleases. In TFAM overexpressing cells, a complete Y-arc was easily detected, even at relatively low exposure. The descending portion of this arc was now the most prominent, although this segment was relatively sensitive to nucleases. The cloud of heterogeneous material was replaced by at least two discrete, slow-moving Y-like arcs, which were also nuclease-sensitive. The replication pause

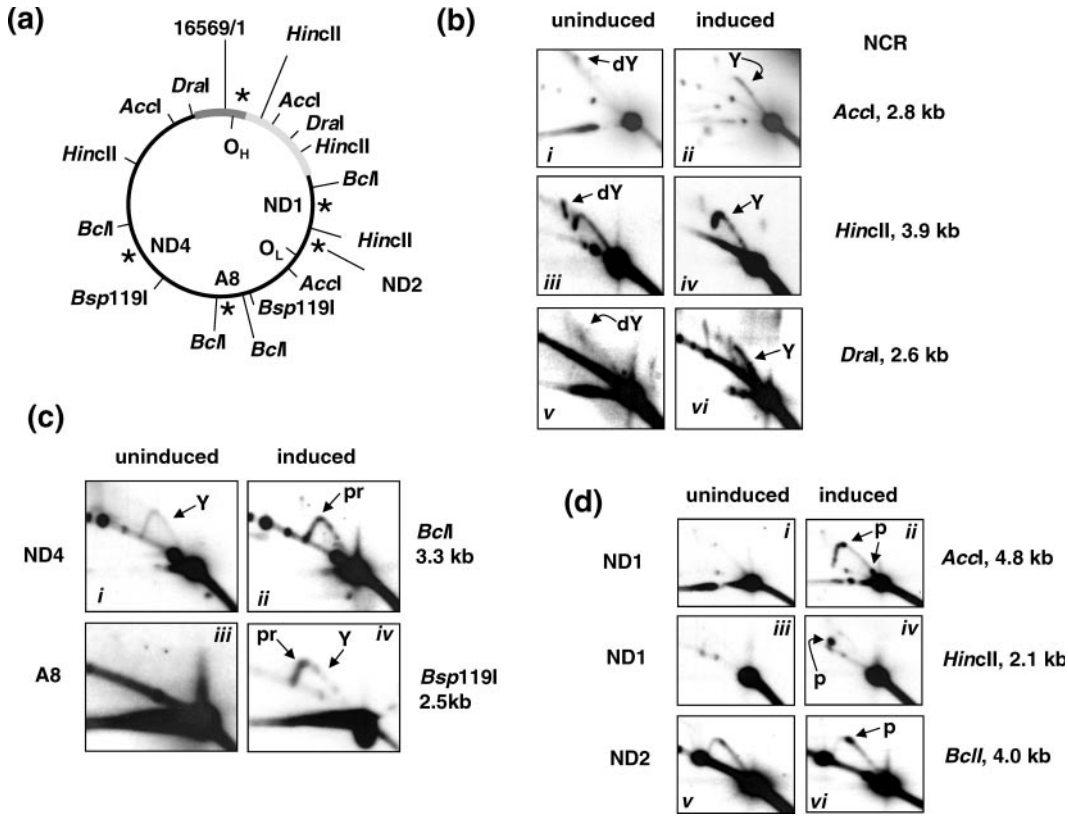

Figure 3. 2DNAGE analysis of S1 nuclease-resistant mtDNA RIs in cells induced to overexpress TFAM-stop. (**a**) Diagrammatic map of human mtDNA, nomenclature as for Figure 2a. (**b–d**) 2DNAGE of mtDNA from uninduced cells and from cells induced to express TFAM-stop for 48 h, analysed using the restriction digests and probes indicated. All samples were treated with S1 nuclease before electrophoresis. Nomenclature as for Figure 2, plus pr, pause region (replication slow-zone). See Supplementary Figure 5 for diagrammatic interpretations. Comparable exposures are shown, to illustrate the general enhancement of S1-resistant RIs, the strengthening of pause sites and regions and the decrease in termination intermediates. Note that bubble arcs are not visible at these exposures following S1 nuclease treatment.

in ND1 was prominently detected after S1 nuclease treatment.

The major NCR (Figure 2c), containing the principal sites of replication initiation and termination, showed more subtle changes, affecting most obviously the termination region. In uninduced cells, the most prominent species were termination intermediates lying on or close to the apex of the X-arc, plus a heterogeneous cloud of material migrating at high molecular weight which, after nuclease treatment, was either modified (RNaseI or H) or abolished (S1). The ascending portion was the most prominent portion of the partial Y arc and this was also sensitive to ribonucleases, as was the bubble arc. In TFAM overexpressing cells the bubble arc was barely visible even on long exposure and the descending portion of the partial Y arc, leading to the termination site, was strongly enhanced. However, termination intermediates lying on the X-arc were less prominent, especially after S1 nuclease treatment. Heterogeneous, high molecular weight, S1-sensitive material was less dispersed and its migration less affected by ribonucleases.

We next analysed the nature of fully double-stranded (i.e. S1 nuclease-resistant) RIs around the genome in further detail, under conditions of TFAM overexpression (Figure 3). Different restriction digests (Figure 3b) confirmed that TFAM overexpression led to an accumulation of material on the standard Y arc in the region approaching the terminus at O_H, with a corresponding loss of termination intermediates in which the fork had entered the fragment from the other end and stalled at the terminus prior to resolution.

A number of pause sites or regions were strongly enhanced, in addition to the strong pause in ND1 (Figure 3d). These included the pause at O_L (Figure 3d), several discrete sites in the regions of ND4, ND3, COXIII, A6 and A8, most of the COXII gene (Figure 3c) and a broad region of the ND5 gene (Figure 2c). Conversely, the abundance of 7S DNA was diminished by TFAM overexpression (Figure 6).

RNAi knockdown of TFAM expression produced more subtle effects on RIs (Figure 4). RIs resistant to S1 nuclease were little altered (Figure 4c), but ribonuclease-sensitive 'clouds' of heterogeneous, nuclease-sensitive material were somewhat enhanced, especially in the rDNA region (Figure 4c) or showed altered mobility. The clearest transformation was seen in the origin/termination region encompassing O_H, where there was an increase in the abundance of double-Y termination intermediates lying on the X-arc, relative to the bubble and partial Y arcs (Figure 4b), an opposite result to that produced from TFAM overexpression, which diminished the abundance of termination intermediates (Figures 2 and 3).

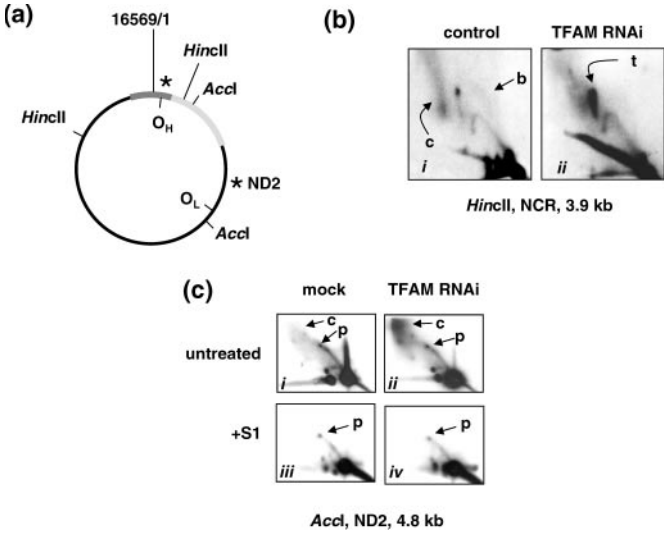

Figure 4. 2DNAGE analysis mtDNA RIs in cells treated with TFAM-specific siRNAs. (**a**) Diagrammatic map of human mtDNA, nomenclature as for Figure 2a. (**b**) and (**c**) 2DNAGE of mtDNA from control (or mock-transfected) cells and from cells treated with TFAM siRNAs for 24 h, using the restriction digests and probes indicated, with or without additional S1 nuclease treatment as shown. Nomenclature as for Figure 2. See Supplementary Figure 5 for diagrammatic interpretations. Comparable exposures are shown, to illustrate the general enhancement of termination intermediates and of the cloud of nuclease-sensitive material migrating at high molecular weight.

TFAM over-expression shows similarities with ddC treatment

Arcs of fully double-stranded, ribonuclease-insensitive RIs should be generated by true strand-coupled replication. However, they could also arise from maturation of ERIOLS type intermediates and, if so, should be enhanced where leading-strand synthesis has stalled randomly or has been drastically slowed (i.e. if the rate of lagging-strand maturation is now comparable with that of fork progression). The effects of TFAM overexpression could thus be interpreted either as a switch to strand-coupled-type replication or as a general slowing in progression of the replication fork, such that maturation of the lagging strand now occurred as fast as the fork progressed.

To address this issue we compared the effects on mtDNA RIs of TFAM over-expression with treatment of cells with the replication inhibitor dideoxycytidine, ddC (Figure 5). After conversion to ddCTP this drug produces repeated chain termination events during mtDNA replication, requiring removal of the incorporated dideoxynucleotide by exonuclease action or recombination, thus greatly slowing down the overall rate of fork progression and leading to mtDNA depletion. As shown in Figure 5, ddC treatment induced rather similar transformations in the pattern of mtDNA RIs as those brought about by TFAM overexpression: progressive and dramatic reduction in ERIOLS-type RIs, a corresponding increase in fully double-stranded RIs of the strand-coupled type (Figure 5d) and the disappearance of termination intermediates (Figure 5b, panels i–iii). Removal of the drug rapidly induced a burst of mtDNA replication but with intermediates remaining initially of the strand-coupled type and with a delayed re-appearance of termination intermediates (Figure 5b, panel vii). However, both during and following ddCTP treatment, the enhancement of RIs of the strand-coupled type was more general than that resulting from enhanced TFAM expression, rather than being concentrated in the rDNA region and at replication pause sites (e.g. compare Figure 5e, panels ii and iii).

Alterations to TFAM expression modify mtDNA topology

The effects of TFAM overexpression on mtDNA replication might reflect TFAM-induced changes in transcription or in the overall organization of mtDNA. To test whether modulation of TFAM expression affects mtDNA topology or organization, we analysed uncut mtDNA from TFAM-induced and uninduced cells, both before and after treatment with various DNA-modifying enzymes (Figure 6). In uninduced cells, most of the mtDNA migrated either as relaxed circles or in high molecular weight catenated forms that were sensitive to topoisomerase IV but not topoisomerase I. A pronounced smear of material was also visible in the high molecular weight region of the gel. Induced TFAM over-expression resulted in a pronounced shift towards monomeric supercoils, with much less catenated mtDNA, including the high molecular weight smear, as well as creating novel junctional forms that were resolved by phage T7 gp3 endonuclease. Despite these differences, the residual products from combined treatment with topoisomerase IV and T7 gp3 were strikingly similar, when comparing TFAM-induced and uninduced cells. ddC treatment also resulted in a shift away from catenated forms in favour of monomeric circles, both relaxed and supercoiled (Supplementary Figure 6). TFAM knockdown by RNAi produced more subtle changes in mtDNA topology (Figure 6), with an increased level of one particular high molecular weight species (arrowed in Figure 6) and of linear molecules. The arrowed species corresponded in mobility with a catenated form which could be enhanced in control cell mtDNA by treatment with T7 gp3 endonuclease and thus might represent an abortive termination product.

Finally, we analysed the effects of various treatments on the steady-state level of 7S DNA. Both ddC treatment [(40); Supplementary Figure 6] and TFAM overexpression (Figure 6b) resulted in a substantial drop in the amount of 7S DNA relative to other forms of mtDNA, whereas TFAM knockdown resulted in a small increase in 7S DNA (Figure 6b).

DISCUSSION

In this study we investigated the effects on mtDNA replication of modulating TFAM expression in cultured human cells. Sustained over-expression, resulting in a >2-fold increase in the ratio of TFAM to mtDNA, greatly enhanced the steady-state levels of RIs of the strand-coupled type, with corresponding depletion of ribonucleotide-rich, ERIOLS-type RIs. This was accompanied by decreased mitochondrial transcription, depletion of 7S DNA and of replication-termination intermediates, reduced copy number and decatenation of mtDNA. TFAM knockdown in the same cell background also produced copy number depletion,

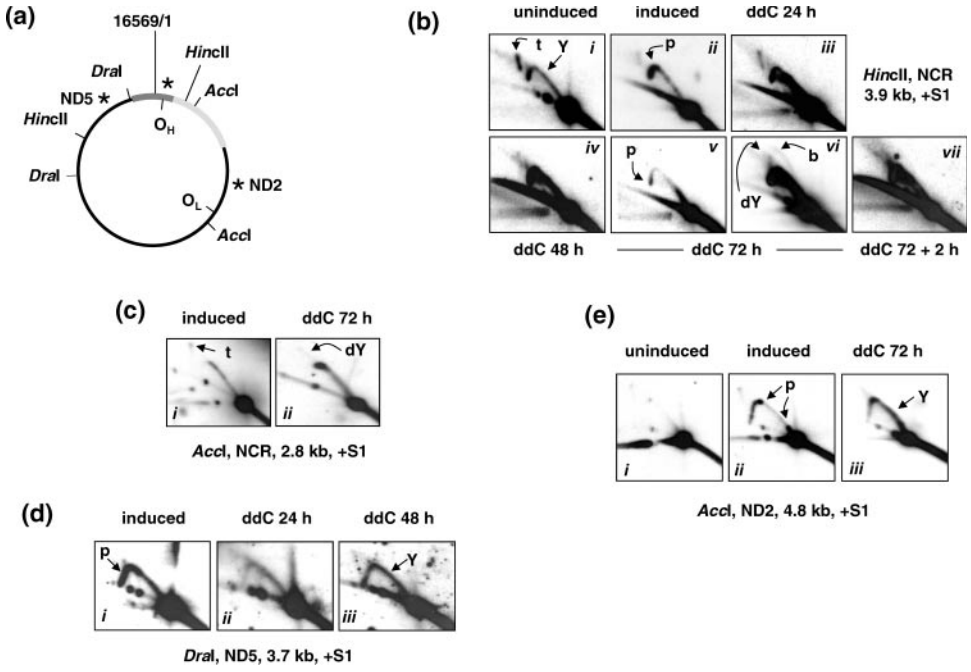

Figure 5. 2DNAGE analysis of RIs in cells induced to overexpress TFAM-stop or treated with ddC. (**a**) Diagrammatic map of human mtDNA, nomenclature as for Figure 2a. (**b**–**e**) 2DNAGE of mtDNA from uninduced cells, cells induced to express TFAM-stop for 48 h or HEK293T cells treated with ddC for the indicated times (72 + 2 h meaning 72 h of treatment followed by 2 h of recovery in fresh medium). Restriction digests and probes as indicated. All samples were treated with S1 nuclease before electrophoresis. Nomenclature as for Figure 2. See Supplementary Figure 5 for diagrammatic interpretations. Exposure times vary, as needed to reveal the main features of arcs of RIs. Note the similar effects of TFAM overexpression and ddC treatment: general enhancement of S1 nuclease-resistant RIs, suppression of termination intermediates. Panel vi of part (c) is a longer exposure of panel v.

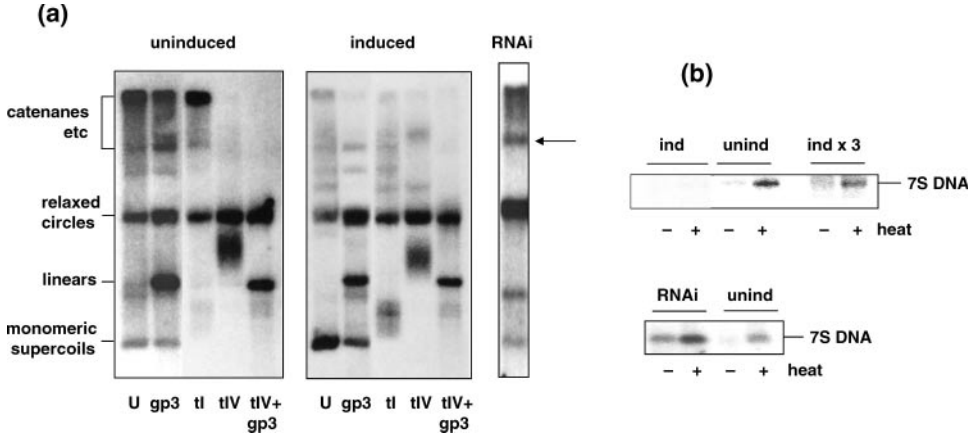

Figure 6. Effects of TFAM overexpression and knockdown on mtDNA topology. One-dimensional agarose gel blots, hybridized with O_H probe. (**a**) mtDNA from uninduced cells and from cells induced to overexpress TFAM-stop for 48 h, fractionated on a 0.4% agarose gel run in TBE. Only the high molecular weight portion of the gel is shown. Samples were either untreated (U) or treated with T7 gp3 endonuclease (gp3), topoisomerase I (tI), topoisomerase IV (tIV) or topoisomerase IV plus T7 gp3 endonuclease. Identity of the main topoisomers was inferred from enzymatic sensitivity and confirmed by other treatments (data not shown). DNA from cells treated with TFAM-specific siRNAs (RNAi) for 24 h was run on a separate gel. (**b**) MtDNA from TFAM-induced, uninduced and siRNA-treated cells, fractionated on 0.4% agarose gels run in TBE. Only the low molecular weight portion of each gel is shown. First 4 lanes of upper panel are equally exposed, whereas the right-most two lanes are ~3-fold overloaded, to reveal the presence of 7S DNA in induced cells. Samples were either heated for 2 min at 95°C (+) or left unheated, as indicated.

but with different alterations to mtDNA topology, no systematic effects on transcription and, apart from a strong enhancement of termination intermediates, virtually no change in the pattern of RIs. The findings suggest that TFAM can influence mtDNA replication and copy number in several different ways.

TFAM as a structural protein of the mitochondrial chromosome

Previous views of TFAM as a copy number regulator have assumed that the ratio of TFAM:mtDNA is invariant, so that changes in the rate of TFAM synthesis determine the amount of mtDNA present in the cell (10,14,30,31).

However, the observation of a reduced ratio of TFAM protein to mtDNA during mtDNA reamplification following EtBr treatment (32) suggests that the TFAM:mtDNA ratio is not invariant and may indeed be subject to regulation, affecting mtDNA copy number in ways distinct from a simple titration model.

The present study offers support to the latter view, by confirming that an increased ratio of TFAM protein to mtDNA can be sustained in human cells and has systematic, but opposite effects on mtDNA copy number than would be predicted by the simple titration model. Moreover, whereas most experiments hitherto conducted on TFAM have employed highly non-physiological tools, such as cells treated with EtBr, heterologous expression of a human protein in mice or of a truncated TFAM variant never seen in nature, we observed alterations in mtDNA replication and copy number resulting from increased expression of a protein identical to endogenous TFAM. The fact that we were able to achieve and sustain an increased ratio of TFAM to mtDNA contradicts the titration model, but is consistent with the results of studies using *in organello* footprinting (41–44), which indicate that protein binding to mtDNA is not uniform and thus that there are sites ordinarily unoccupied by protein where over-expressed TFAM can potentially bind.

The effects of TFAM over-expression on RIs were qualitatively very similar to those brought about by treatment with the drug ddC which, following conversion to ddCTP, is assumed to be a potent inducer of repeated replication stalling via premature DNA chain termination. ddC treatment also resulted in a large increase in the steady-state levels of nuclease-resistant RIs of the strand-coupled type from all around the mitochondrial genome, with concomitant loss of termination intermediates. Although the effects of these treatments were not absolutely identical, with TFAM over-expression generating a subtly different pattern of such RIs, especially in the rDNA region and in the vicinity of pause sites, their overall similarity strongly suggests that the main effect of TFAM is, like ddC, to provoke a substantial decrease in the net rate of DNA synthesis. This may reflect an increased compaction of mtDNA when the ratio of TFAM protein to mtDNA is increased, such that decondensation of the nucleoid becomes rate-limiting for fork progression. The TFAM-specific transformations of RIs may reflect different degrees of compaction and inhibition of fork progression in different regions of the mitochondrial genome, depending on their affinity for TFAM and other proteins.

The increased abundance of strand-coupled RIs may thus be due to maturation of ERIOLS-type intermediates, which we assume normally to be a slow step compared with the rate of fork progression. If the latter is slowed by increased compaction, the rate of maturation may become comparable with it. Alternatively, if the two classes of RI represent entirely different modes of DNA replication, ERIOLS type replication might be unable to use a highly compacted mtDNA template, leaving only strand-coupled replication (with a less discrete origin, as implied by Figure 2b). Initiation of bidirectional, strand-coupled replication in many bacterial plasmids is stimulated by or dependent on, DNA-bending proteins functionally related to the HMG superfamily (45). Excess TFAM may thus lead to copy number depletion by enforcing a switch to an inherently slower replication mode or simply by suppressing the alternative mode.

Exactly the same transformations of RIs were produced by transient expression of TFAM as were produced by inducible expression of either natural or C-terminally MycHis-tagged TFAM. However, the two inducible variants brought about copy number depletion with markedly different kinetics (Figure 1). Copy number depletion may thus not be due entirely to a change in the rate of fork progression, but involve also another TFAM-related process with which the C-terminal tag may interfere. Such an effect could, however, be indirect. The amount of TFAM-MycHis protein rose much more steeply than TFAM-stop during induction (Figure 1, Supplementary Figures 1 and 2), suggesting that it may escape physiological turnover mechanisms. This is supported by the observation (Supplementary Figure 2) that TFAM-MycHis almost completely replaced endogenous TFAM during induction. The fact that sustained overexpression of natural TFAM is comparatively difficult to achieve may reflect a natural homeostatic mechanism, whereby TFAM levels can modulate copy number only within certain limits.

Another possibility is that, as for other HMG proteins (46), the C-terminal tail of TFAM recruits or interacts with other nucleoid proteins. It is already implicated in interactions with specific components of the transcriptional apparatus (47). The epitope tag may therefore disturb interactions with other nucleoid proteins involved in copy number homeostasis. Copy number derangement produced in cells or mice by C-terminally truncated or heterologous TFAM variants (14,30,31) may prove to be due to loss of such regulation, rather than by the titration model inferred previously.

The fact that RNAi knockdown of TFAM results in copy number depletion without substantial changes in the patterns of RIs is further evidence that at least one other TFAM-dependent process is critical for mtDNA maintenance. One obvious possibility already mentioned is the compaction of nascent mtDNA into TFAM-containing nucleoid structures, in the absence of which the newly replicated mtDNA may simply be unstable. Other possibilities are discussed below.

TFAM as a regulator of mitochondrial transcription

Although TFAM was originally identified and named on the basis of its being essential for mtDNA transcription, previous studies have shown that excess TFAM, supplied either to a reconstituted *in vitro* system (9) or produced by sustained overexpression in HEKcultured cells (27), results in a paradoxical suppression of transcription. Our own observations are consistent with this (Figure 1, Supplementary Figures 1 and 2).

A drop in transcriptional activity could, conceivably, underlie the switch in DNA replication mode that favours the generation of strand-coupled RIs. Although the decrease in transcriptional activity which accompanies TFAM overexpression appears modest, according to the data of Figure 1 (Supplementary Figures 1 and 2), the steady-state level of ND3 mRNA is a relatively insensitive measure of the actual transcription rate. The mechanism by which ERIOLS-type RIs are generated remains unknown. However, since on 2DNAGE they include heterogeneous 'clouds' of material sensitive to both RNaseI and RNaseH which are suppressed

by TFAM overexpression (Figure 2), it is possible that they could arise by a mechanism involving either preformed RNA or nascent transcripts. Transcriptional suppression by excess TFAM, rather than over-compaction of the nucleoid, may therefore be the mechanism driving replication towards the slower, strand-coupled mode. In support of this, the segment of the genome most affected by TFAM overexpression is also the most heavily transcribed region (rDNA), in which discrete RIs are ordinarily hard to detect (Figure 2d), but became prominent when TFAM was overexpressed.

Whereas copy number depletion brought about by TFAM overexpression was accompanied by a clear drop in transcription, transcription per template molecule appeared unchanged when copy number was depleted by TFAM knockdown. Copy number regulation may therefore be independent of transcription. However, transcription was more severely affected by overexpression of epitope-tagged TFAM than natural TFAM and also provoked a more rapid drop in mtDNA copy number. The issue of a relationship between the transcriptional activity of TFAM and copy number control thus remains open.

TFAM overexpression leads to reduced levels of 7S DNA, which might reflect decreased transcriptional activity at the light-strand promoter and/or enhanced resolution of D-loop forms *in vivo*, another known property of TFAM [(19), see also following section]. If D-loops represent a precursor step in DNA replication, as proposed by the orthodox model, their depletion by TFAM overexpression may be crucial in bringing about copy number reduction or in inducing a switch to strand-coupled replication. On the other hand, the increased level of 7S DNA resulting from TFAM knockdown shows clearly that the D-loop form is not sufficient to maintain a high copy number.

TFAM as an enhancer of replication pausing

Replication pauses are well documented in both bacteria and eukaryotes, as well as in plasmids and mtDNA (4,48). However, their roles in DNA homeostasis are unclear, except where they function as definitive terminators. In human mtDNA, prominent pauses occur at the so-called termination-associated site delimiting 7S DNA, at O_L and in the region immediately downstream of rDNA, within the ND1 gene (Figures 2 and 3). One possibility is that, as in yeast rDNA, these pauses are the signatures of proteins which bind at specific sites to facilitate the passage of oppositely moving replication and transcription complexes (49) or simply markers of collision sites, as in bacteria (50), at which replication finally resumes after dissociation of the transcriptional machinery (51). Although TFAM has only a low sequence-specificity for DNA-binding, it may enhance such pauses by promoting DNA-bending, as proposed for the protein Sap1p at one of the replication pause sites in *Schizosaccharomyces pombe* rDNA (52). Such bending may also facilitate the binding of other, more sequence-specific DNA-binding proteins, which directly function in fork arrest. Both copy number depletion and transcriptional inhibition following TFAM overexpression may therefore be due, at least in part, to its effect as a strong enhancer of replication pausing.

TFAM as a cofactor in junctional resolution

TFAM overexpression and TFAM knockdown produced opposite effects on the abundance of termination intermediates. Whereas these were depleted by TFAM overexpression (or ddC treatment), they were strongly enhanced by TFAM knockdown (Figures 3–5). A simple interpretation is that resolution of these forms is a late and slow step in mtDNA replication, which is in some way dependent on the supply of TFAM. Under conditions of TFAM overexpression, either that step is facilitated by increased loading of TFAM onto the nascent DNA or the rate of fork progression is slowed down so much that resolution of termination intermediates is no longer rate-limiting. Under conditions of TFAM 'starvation' this resolution step would conversely be inhibited and this may be the primary reason for copy number depletion following TFAM knockdown.

TFAM is already known to have binding preference for junctional structures (17), to facilitate the resolution of D-loops (19) and to bend DNA in a manner analogous with bacterial proteins such as HU and IHF. The exact molecular structure of mtDNA replication-termination intermediates is unclear, although it cannot simply comprise two oppositely moving forks that have almost met, since such a structure would be thermally unstable unless the unreplicated region between them were at least 50 bp, in which case they would not lie precisely on the X-arc on 2DNAGE gels. One possibility is that they are held together by hemicatenation after ligation of at least one strand, although such forms should be S1 nuclease-sensitive (53). Another is that they are converted to true Holliday junctions. A third option is that they contain a single or even double chicken-foot structure.

Whatever their precise nature, a complex enzymatic machinery should be required both to form and to resolve them, in which TFAM is a plausible player, both because of its DNA-binding preferences and bending properties and by its putative ability to recruit other proteins to the DNA. DNA-bending is required for the protein-based partition systems of low copy-number bacterial plasmids such as P1 (54) and binding sites for the DNA-bending proteins H-NS and FIS appear to be clustered around the replication terminus in *Escherichia coli* (55). HMG and related proteins are widely implicated as cofactors in recombination. For example, mammalian HMGB1 facilitates V(D)J recombination by the RAG proteins (56). In bacteria, HU is essential for efficient homologous recombination (57) and IHF is required for the action of lambda integrase (58). HMG proteins are able to facilitate recombination even in heterologous systems [see Ref. (59)]. In yeast mitochondria, overexpression of Abf2p promotes the formation of recombination intermediates (60). DNA-bending by TFAM may facilitate analogous processes in human mtDNA.

A rather different interpretation would also be consistent with the 2DNAGE data, namely that TFAM overexpression leads to a high frequency of strand breakage at O_H. This would account both for the relative paucity of bubble arcs in O_H-containing fragments, (Figure 2b and c), for the corresponding increase in simple Y arcs and for the loss of termination intermediates (Figures 2c, and 3b). Such strand-breakage is very unlikely to be an extraction artefact, since these differences from control cells were seen in all of the

many DNA preparations analysed. If this interpretation is correct, it may imply a need for true recombination reaction to resolve daughter molecules, which could be much slower than the usual termination/resolution step. Copy number depletion might therefore be due to interference with termination when TFAM is either overexpressed or downregulated.

In addition, TFAM overexpression resulted in apparent decatenation of mtDNA, whereas TFAM knockdown increased the level of linear molecules and of one decatenated species that could also be generated in control cell mtDNA by treatment with T7 gp3 endonuclease, a promiscuous junctional resolvase. It is tempting to suggest that these species may be aberrant breakdown products resulting from failure of the normal termination step under conditions of TFAM deficiency. The loss of catenated species in favour of monomeric supercoils under conditions of TFAM overexpression may again reflect the altered kinetics of mtDNA replication, since it too was brought about by ddC treatment. Alternatively an overcompacted state of mtDNA resulting from excess TFAM may directly prevent catenation or stimulate decatenation, as is known for the *E.coli* HU protein acting on circular plasmid molecules (61). Decatenation might also result from frequent strand-breakage at O_H. Under conditions of decreased copy number, decatenation should be important for dispersal of newly replicated mtDNAs into nucleoids, to restrain mitotic segregation of possibly deleterious mtDNA sequence variants. In yeast, the TFAM homologue Abf2p is required for efficient parsing of mtDNA into nucleoids (62), although this process appears to involve recombination rather than decatenation.

Physiological role of TFAM as a mtDNA copy number regulator

Our findings indicate that, at least in this particular proliferating cell background, TFAM expression is finely poised. Any marked deviations from what appears to be an optimal expression level provoke alterations to mtDNA replication that result in decreased mtDNA levels.

Previous studies of the transcriptional regulation of the TFAM gene indicate that it is sensitive to signals connected with metabolite supply (63) and redox stress, via the phosphorylation of NRF-1 (64) and to proliferative, differentiation-linked and environmental signals via the co-activators PGC1 and PRC (65–67) and the transcription factor Myc (68). This raises the issue of whether there is an additional homeostatic, mechanism to fine-tune TFAM expression to the physical state of mtDNA, which can be circumvented by the manipulations we carried out. A retrograde signalling pathway of this type might serve to ensure that disturbances in mtDNA replication or segregation do not lead to a copy number catastrophe. Alternatively, the level of TFAM expression and possibly also its post-translational modification (69), may function *in vivo* to accelerate or decelerate mtDNA replication, thus constituting a key determinant of copy number.

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Alterations to the expression level of mitochondrial transcription factor A, TFAM, modify the mode of mitochondrial DNA replication in cultured human cells

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SUPPLEMENTARY DATA

Legends to Supplementary Figures

Supplementary Figure 1

Effects of induced expression of TFAM-stop. Mitochondrial proteins, DNA and RNA were analysed from Flp-In™ T-Rex™ -293 cells stably transfected with the TFAM-stop construct, induced over the times indicated. In each case, error bars indicate means ± SEs from at least three independent experiments. (a) Western blot probed for TFAM protein, stripped and reprobed for α-actinin as loading control. Bar chart shows TFAM chemiluminescence signals normalized first against those of α-actinin, then against that the value for uninduced cells (t=0). (b) Southern blot probed for nuclear and mitochondrial DNA, using ND4 and 18S rDNA probes, respectively. Bar chart shows the ratio of hybridization signals, normalized against the mean value for uninduced cells. (c) Q-PCR analysis of mtDNA copy number on the same samples, again normalized to the mean value for uninduced cells. Note that this assay measures all mtDNA, not just the full-length mtDNA detected in Southern blots, i.e. it includes also replication intermediates. Conversely, mtDNA which is amplified poorly, e.g. due to ribosubstitution, may be under-quantified by this method. Southern blotting, on the other hand, measures only mature mtDNA, but is potentially subject to artefacts arising from minor variations in sample quality. The two methods in fact gave slightly different results. The transient increase in copy number in the first hours of TFAM over-expression seen by Q-PCR. may, indeed, be due to an increased level of replication intermediates. To minimize any artefacts introduced by one or other method, the mean of the two values was used to calculate the data plotted in Fig. 1. (d) Northern blot probed for mitochondrial ND3 mRNA, stripped and reprobed for 5S rRNA. Bar chart shows the ratio of hybridization signals, normalized against the mean value for uninduced cells.

Supplementary Figure 2

Effects of induced expression of TFAM-MycHis. Mitochondrial proteins, DNA and RNA were analysed from Flp-In™ T-Rex™ -293 cells stably transfected with the TFAM-MycHis construct, induced over the times indicated. In each case, error bars indicate means ± SEs from at least three independent experiments. (a) Western blot probed for TFAM protein, stripped and reprobed for α-actinin as loading control. The identity of the band denoted as the TFAM-MycHis protein (TFAM-mh) was confirmed by probing with anti-

Myc monoclonal antibody. Bar chart shows total TFAM chemiluminescence signals (i.e. endogenous TFAM plus transgenic TFAM-MycHis) normalized first against those of α-actinin, then against the value for uninduced cells (t=0). Note that endogenous TFAM declines as overexpressed TFAM-MycHis increases, indicating that TFAM levels are also regulated translationally or post-translationally, most likely at the level of incorporation into protein-DNA complexes. (b) Southern blot probed for nuclear and mitochondrial DNA, using ND4 and 18S rDNA probes, respectively. Bar chart shows the ratio of hybridization signals, normalized to the mean value for uninduced cells. (c) Q-PCR analysis of mtDNA copy number on the same samples, again normalized to the mean value for uninduced cells. See legend to Supplementary Fig. 1 for discussion of the issues relating to the two methods used to estimate mtDNA copy number changes. (d) Northern blot probed for mitochondrial ND3 mRNA, stripped and reprobed for 5S rRNA. Bar chart shows the ratio of hybridization signals, normalized against the mean value for uninduced cells. (e) Western blot of mitochondrial protein extracts from TFAM-MycHis after 24 h of induction, sub-fractionated into pellet (Pel) and supernatant (Sup) fractions, and probed for TFAM. The adjacent tracks are equally loaded in regard to the starting material. Based on chemluminescence, approximately 90% of both the endogenous and the transgenic TFAM fractionate in the pellet, along with mtDNA.

Supplementary Figure 3

Effects of RNAi knockdown of TFAM expression. Mitochondrial proteins, DNA and RNA were analysed from HEK293T cells, following transfection with siRNAs Si2 and Si5, at the times indicated. In each case, error bars indicate means $\pm$ SEs from at least three independent experiments. (a) Western blot probed for TFAM protein, stripped and reprobed for α-actinin as loading control. Bar chart shows TFAM chemiluminescence signals normalized first against those of α-actinin, then against the value for untreated cells (t=0). (b) Q-PCR analysis of mtDNA copy number, normalized to the mean value for untreated cells. (c) Northern blot probed for mitochondrial ND3 mRNA, stripped and reprobed for 5S rRNA. Bar chart shows the ratio of hybridization signals, normalized against the mean value for untreated cells. (d) Western blot of mitochondrial protein extracts from cells treated for 5 d in trial experiments with various combinations of putative TFAM-specific siRNA oligonucleotides. The sequences of the relevant mRNA targets were as follows. Si1: 5´-AAAGAAACCTGTAAGTTCTTA -3´ (np 282-302 of the TFAM mRNA

sequence), Si3: 5´-AAAGAAAAAAATATATCAAGA-3´ (np 414-434), Si5 5´-AAAGAAGAGATAAGCAGATTT-3´ (np 463-483), Si6: 5´-AAGCAGATTTAAAGAACAGCT-3´ (np 474-494). The final lane shows protein from cells treated with an siRNA directed against mTERF mRNA, as a negative control.

Supplementary Figure 4

Suppression of *de novo* mtDNA transcription in cells induced to express TFAM-MycHis. Cells were cultured in EtBr-containing medium for 72 h, with or without concomitant induction of TFAM-MycHis expression by doxycyclin, followed by replating in fresh medium containing no EtBr. Mitochondrial tRNA levels at different time-points were measured by Northern hybridization and phosphorimaging, with normalization first to 5S rRNA as a loading control, and then to the starting level of the relevant tRNA in uninduced cells on the first day of measurement (24 h after the start of the experiment). Over 5 days of induction, TFAM-MycHis expression reduces mtDNA copy number only by 30-50% (Supplementary fig. 2), therefore the almost complete suppression of *de novo* transcription revealed by this experiment is not due to the absence of template.

Supplementary Figure 5

Explanations and supplementary data for 2DNAGE analyses. (a) Various panels from Figs. 2, 3 and 4 are reproduced, alongside interpretative illustrations of the various classes of replication intermediate assumed to be represented by the arcs indicated. Restriction sites remaining uncut due to ribosubstitution on the lagging strand are indicated by red bars. The clouds of RNAse-sensitive material associated with slow-moving arcs are suggested to have attached tails of nascent RNA, as shown in red. Replication pause regions are shown as filled circles: the one at O_L in yellow, a second one within the ND1 gene in blue. The short 'eyebrow' arc, migrating in the region of the termination intermediates from Fig. 2b, panel ii, is proposed to derive from broken theta molecules forms as shown, although it may, alternatively, comprise dimeric circles. A comprehensive explanation of 2DNAGE methodology and gel interpretation is given in Refs. 70 and 71. (b) Comparison of 2DNAGE analyses of the ND2 region (*Acc*I digest, ND2 probe, see Fig. 2a) in cells overexpressing TFAM-stop or TFAM-MycHis (TFAM-mh) by induction in stably transfected Flp-In™ T-

Rex™ -293 cells, or TFAM-stop by transient transfection of HEK2923T cells, with or without additional enzymatic treatments as shown. Panels v and vi are scaled versions of panels i and ii of Fig. 3d. The patterns of mtDNA RIs are virtually indistinguishable in the three cases of TFAM overexpression, but quite different from those seen in uninduced cells.

Supplementary Figure 6

Effects of ddC treatment on mtDNA topology. DNA samples were analysed as in Fig. 6. ddC treatment was for the times indicated (h), 72 + 2 meaning 72 h of treatment followed by 2 h of recovery in fresh medium etc. The panel showing 7S DNA is a longer exposure of the bottom of the same gel blot.

Additional References for Supplementary Figures

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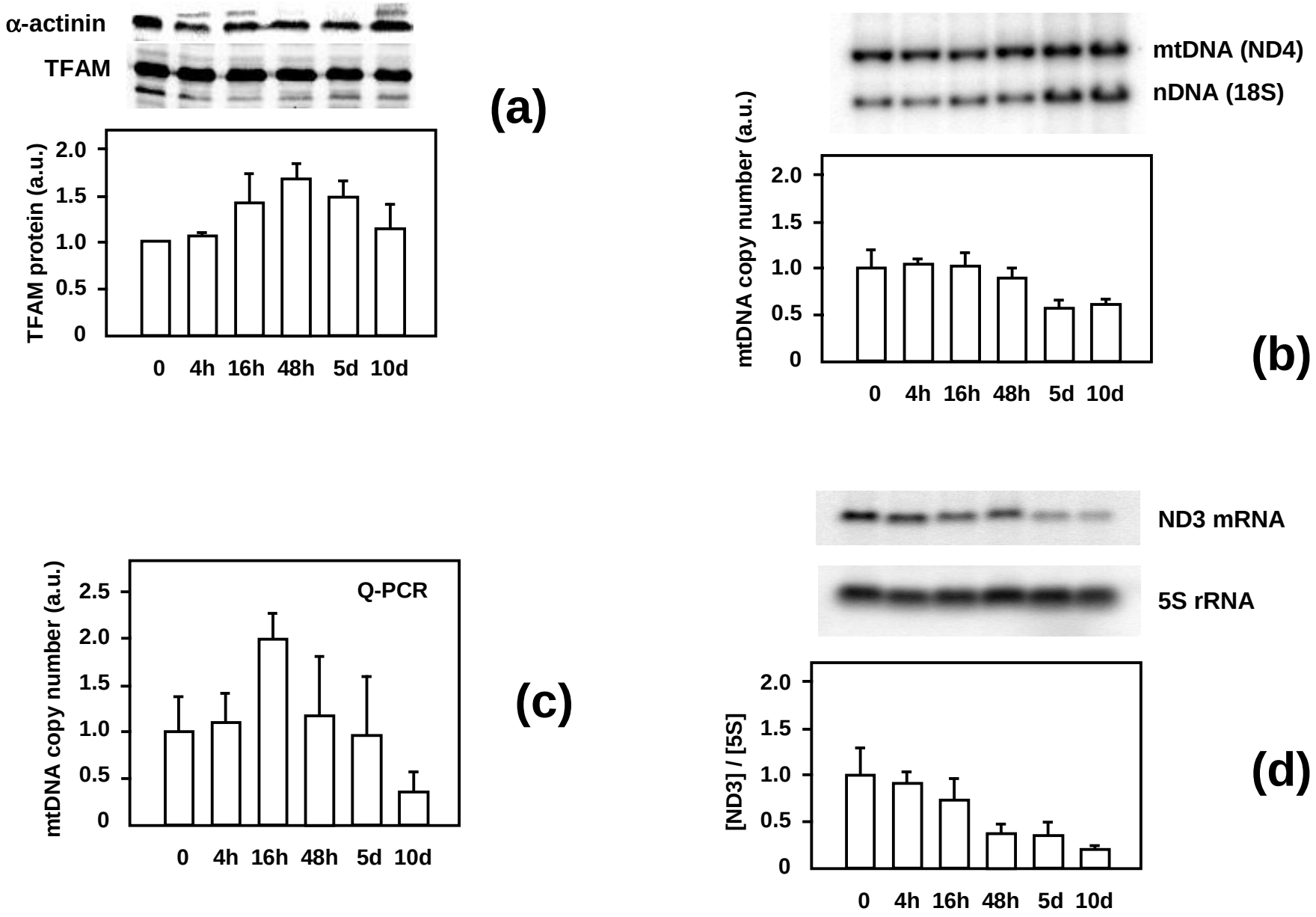
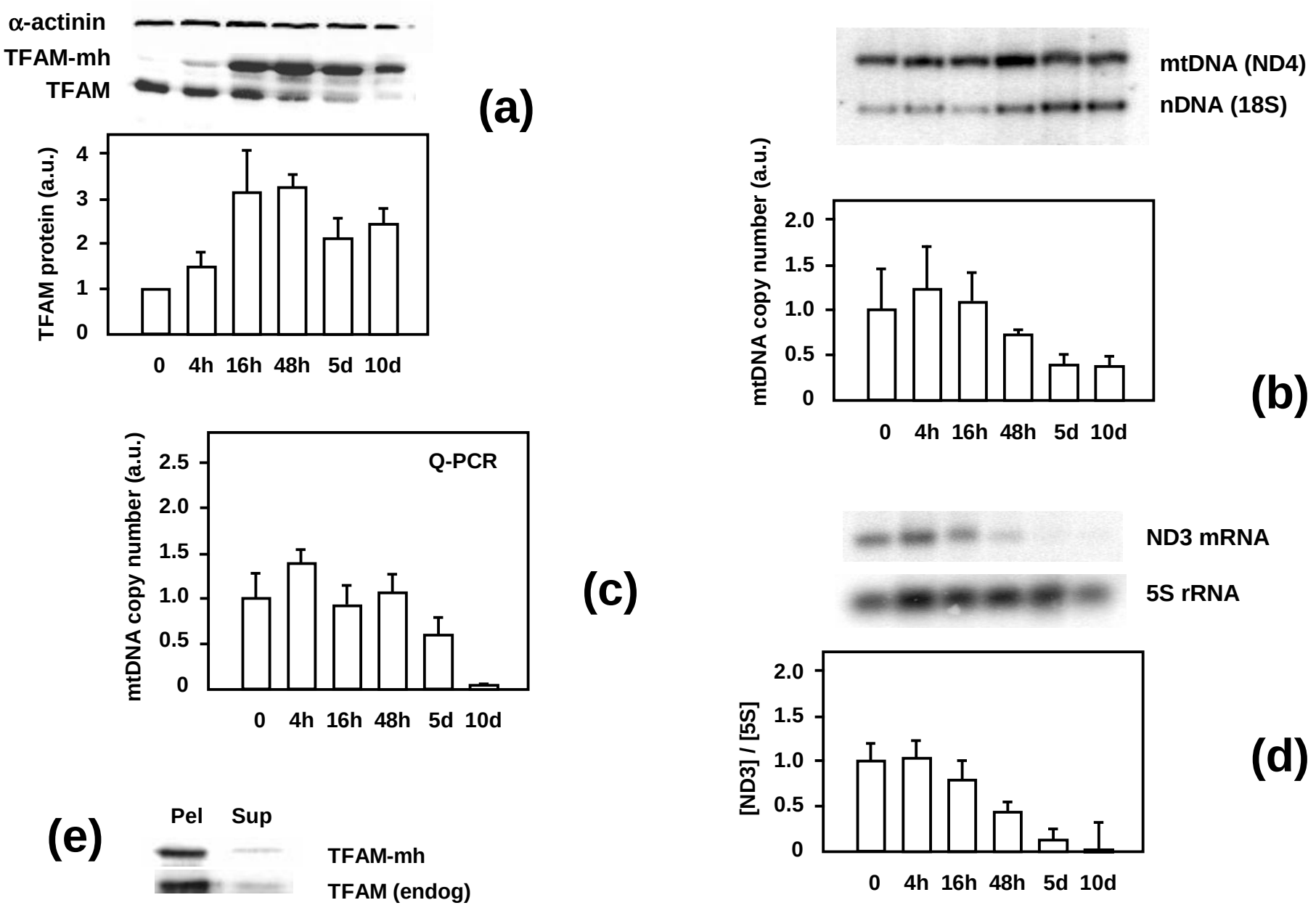
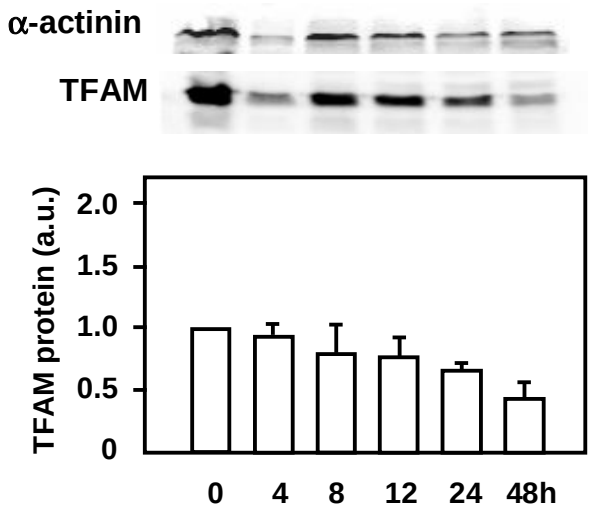


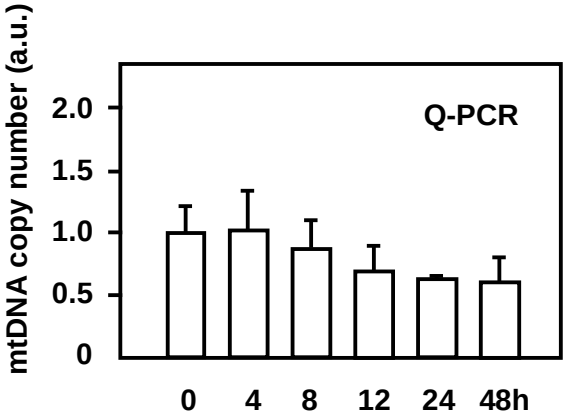
Supplementary Figure 1: Pohjoismäki *et al*



Supplementary Figure 2: Pohjoismäki *et al*



(a)



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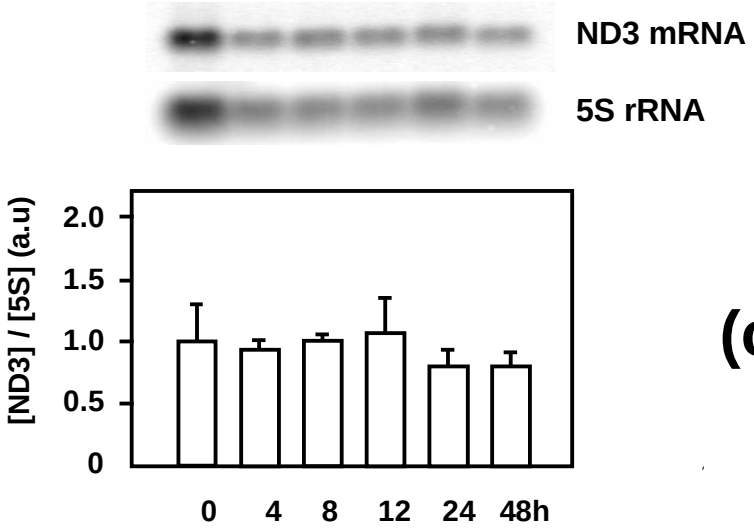

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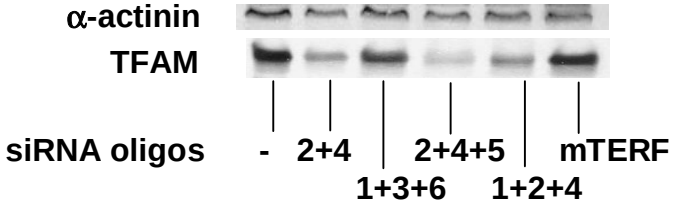
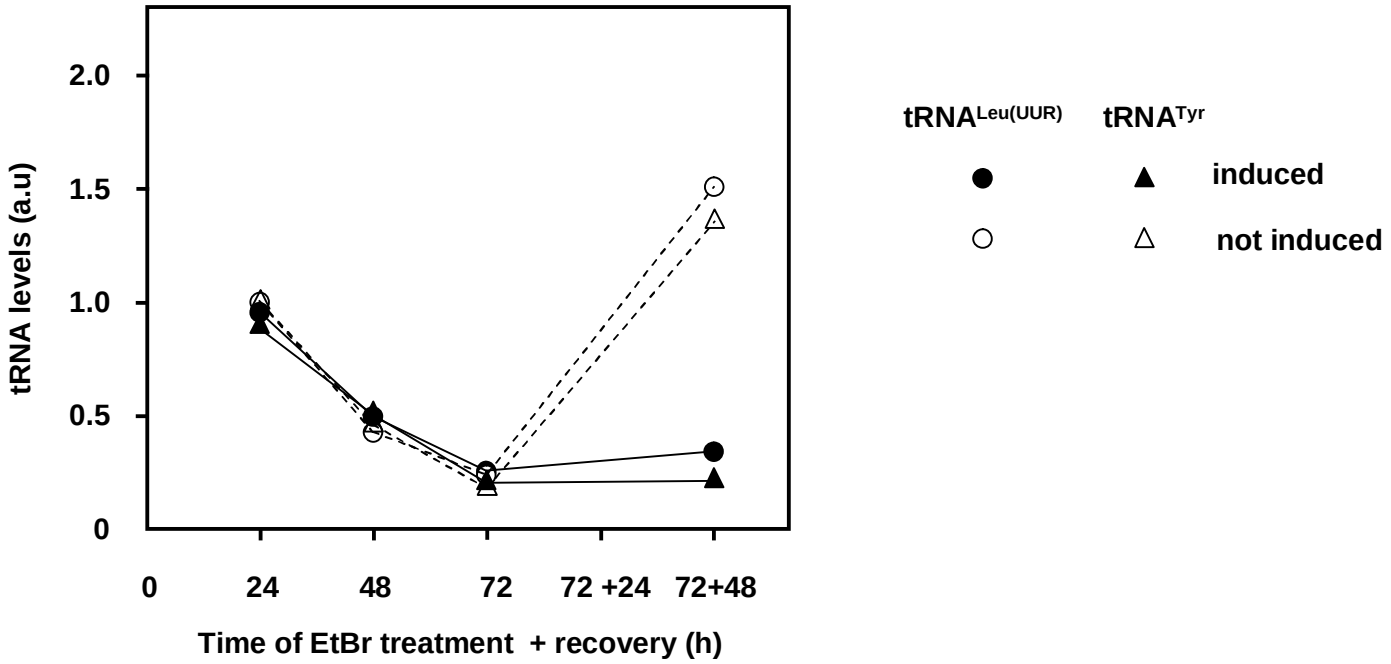


(d)

Supplementary Figure 3: Pohjoismäki *et al*



Supplementary Figure 4: Pohjoismäki *et al*

(a)

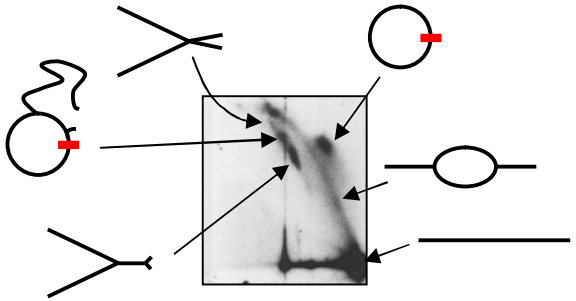


Fig. 2b, panel ii

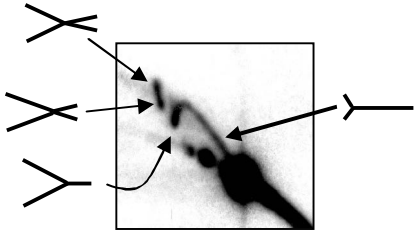


Fig. 3b, panel iii

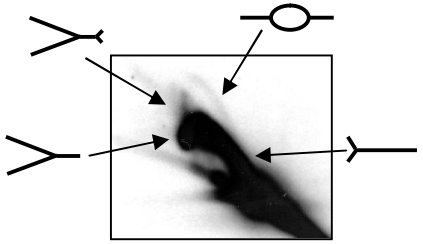


Fig. 5b, panel vi

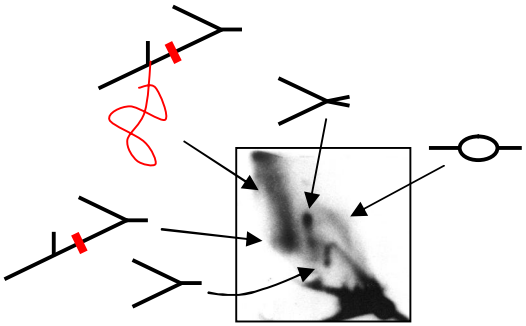


Fig. 2c, panel i

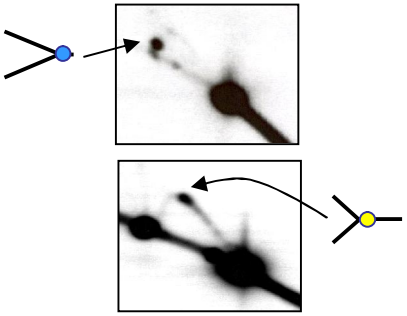


Fig. 3d, panels iv, vi

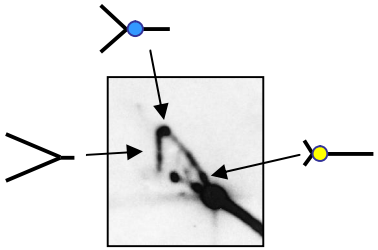


Fig. 2d, panel viii

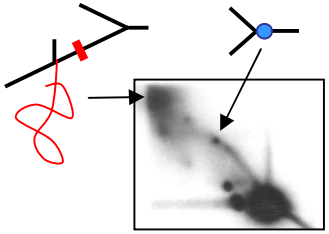
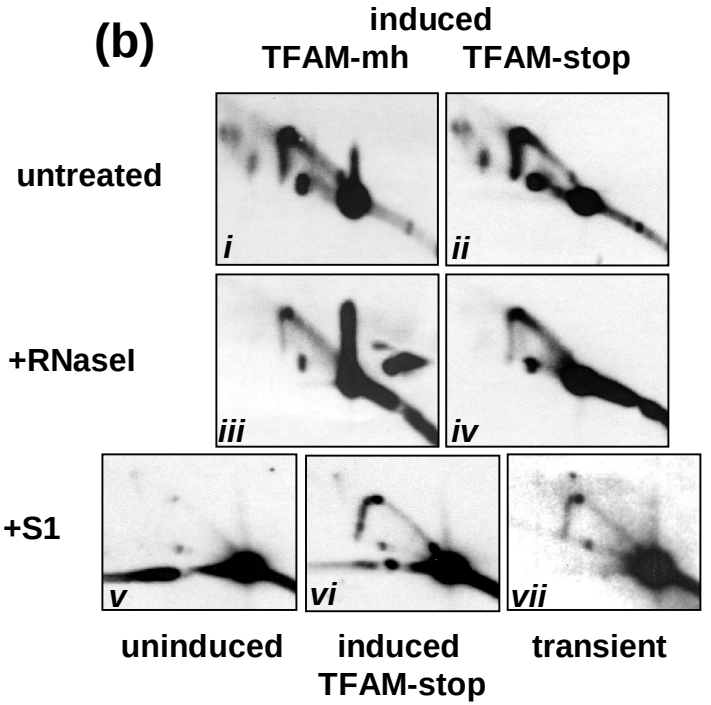


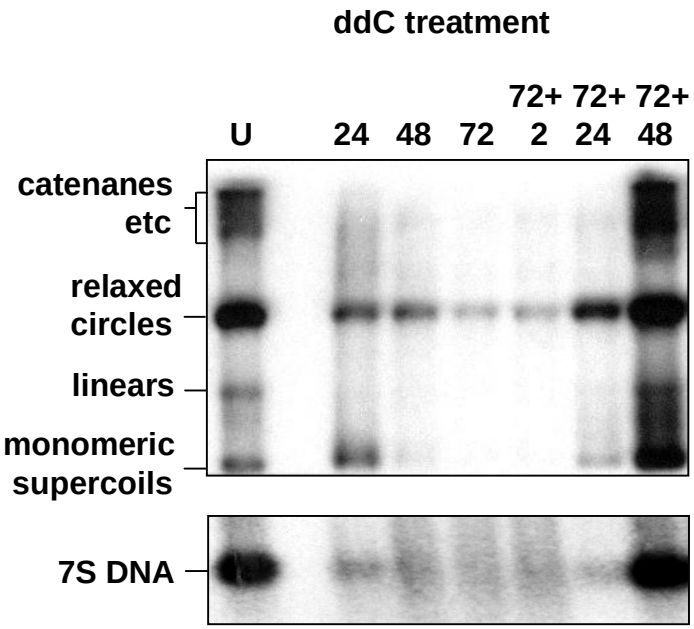
Fig. 4c, panel ii

(b)



*Acc*I, ND2, 4.8 kb

Supplementary Figure 5: Pohjoismäki *et al*



Supplementary Figure 6: Pohjoismäki *et al*